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# Mapping the topographic epitopes of a model antigen

Didrik Paus

A thesis submitted to The Open University in candidature for degree of  
Doctor of Philosophy

March 2003

MRC Laboratory of Molecular Biology / MRC The Protein and Nucleic  
Acid Chemistry Division

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# Declaration

This thesis is 177 pages, comprising 34,000 words and describes work carried out at the MRC Laboratory of Molecular Biology and MRC The Protein and Nucleic Acid Chemistry Division, Cambridge between October 1999 and January 2002. All work described has been carried out solely by myself, with the exception of the contributions of collaborators listed below. None of the work is to this date published. This work has not been submitted whole or in part for any other degree.

Didrik Paus



March 2003

- The mass spectrography of the biotinylated  $\beta$ -lactamase was carried out by Isabelle Lavenir at MRC Laboratory of Molecular Biology, MRC The Centre of Protein Engineering, Cambridge.

- The monoclonal antibodies were made under close supervision and with help from Tone Varaas at the Central Laboratory, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway.

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# Abstract

The thesis describes the development of a method for determining the antigenicity of different regions of the surface of a protein. The method involved the construction of a set of mutant antigens in which cysteine residues had been introduced at different points in the surface of the antigen. Each mutant protein was tethered through the surface cysteine residue and a bifunctional chemical cross-linker to solid phase, allowing the creation of an array of mutant antigens, each oriented so as to expose or to mask different topological regions of the antigen surface. Indeed, as expected, binding of a set of monoclonal antibodies proved sensitive to the orientation of the antigen. By use of this array of oriented antigen it became possible to compare the different regions of the antigen surface for binding to rabbit antiserum, and thereby to create a topological map of the antibody response. When the immunisations were undertaken with use of Freund's adjuvant, the antibody response to  $\beta$ -lactamase was mainly directed against a region centered on a flexible loop. Furthermore it comprised antibodies cross-reactive to both native and denatured protein. By contrast, in the absence of Freund's adjuvant, the antibody response was more evenly distributed, and with few cross-reactive antibodies. This suggested that Freund's adjuvant denatures  $\beta$ -lactamase (as was confirmed experimentally), and that the appearance of dominant epitopes may follow the presentation of denatured protein to the immune system. These observations have implications for understanding the basis for dominance of B-cell epitopes, and also for design of vaccines.

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# Chapter 1

## Introduction

### 1.1 Aim of this work:

The aim of this work is to map the relative distribution of B-cell epitopes on the three-dimensional surface of a protein antigen and to study the relationship between the surface structures and the antigenicity distribution.

### 1.2 The immune response

The immune response can be arranged into two parts, innate immunity and adaptive immunity. Innate immunity is an inherited potential to respond to pathogens and it will not change after a challenge by a pathogen. In contrast, the adaptive immunity, which comprises B and T cells, is able to launch an antigen specific response and retain the antigen specificity long after the initial response. It will upon later rechallenge by the same or a very similar pathogen provide a quicker and more powerful immune response. This specific memory was discovered in 1798 by Edward Jenner [Jenner, 1798] who noticed that individuals who had encountered cow pox were "secure from the infection of the small pox." He also showed that he could vaccinate a young boy against small pox the same way.

### 1.2.1 B cells

B cells are responsible for humoral adoptive immunity through production of antibodies. Antibodies bind extracellular pathogens or toxins and block their ability to infect or destroy body tissue. A second function of antibodies is to opsonise material for clearance by phagocytic cells. The fact that non-cellular substances can protect against foreign antigens was first demonstrated by von Behring and Kitasato in 1890 [von Behring and Kitasato, 1890]. They injected sera from animals that were immunised with and resistant to cholera toxin into normal animals and showed that this protected the latter against the toxin. The "anti-toxines" responsible for the protection are today known as antibodies. In 1947 Fagraeus [Fagraeus, 1947] demonstrated that antibodies are produced by plasma cells (activated B cells). In particular she showed that the number of plasma cells in the spleen increases simultaneously with the amount of circulating antibodies and that tissue from the red pulp, where the plasma cells are located, liberate significant yields of antibodies in cell culture. She also showed that lymph follicles which are rich in lymphocytes, but have no plasma cells, have much lower capacity to produce antibodies.

The mechanism behind the generation of antibodies puzzled investigators for a long time and the research has been driven by several theories. Paul Ehrlich suggested in 1898 a model, "the side-chain theory", to explain the explosive production of antibodies after exposure to antigen [Ehrlich, 1900]. He postulated that cells have a large number of different predefined receptors (side-chains) on the surface and that at least one receptor is able to bind any given antigen. The specific receptor would, when binding to the antigen, be released into the blood stream. New receptors would then be produced to replace the released receptors.

Landsteiner [1936] later showed that the immune system can make antibodies to an apparent unlimited range of antigens. Since no protein structures were known at that time he used chemicals "haptens" coupled to carrier proteins as antigens. Through analysis of the antibodies ability to precipitate the antigens he noticed that antibodies can be

produced against many novel molecules and that the antibodies were able to discriminate subtle differences in the haptens. Ehrlich's theory did not explain how the immune system has enough predefined receptors to respond to such a range of antigens. One explanation was attempted in a model introduced as "the template theory" and refined by Pauling [1940]. It postulated that the antibodies of one predefined sequence fold onto the antigen and essentially that the antibody polypeptide sequence can have many possible conformations with similar folding energy. The antibody therefore uses the antigen as a template to obtain a specific conformation.

But this theory was unable to explain several observations as pointed out by Jerne in 1955 [Jerne, 1955]. In particular, a secondary antigen stimulus provokes a more active production of antibodies than does the primary stimulus and the immune system continues producing antibodies long after the stimulus. Jerne introduced "the natural selection theory". This postulated that a vast repertoire of diversified antibodies is produced and secreted by cells and that binding of antibody to an antigen, followed by phagocytosis of the antibody-antigen complex, triggers production of a large number of copies of the same antibody. Jerne envisaged that the phagocytosing cell duplicated the antibodies and not the cell from which the antibody clone originated. In contrast, Burnet [1957] linked the antigen-antibody recognition to the antibody production by saying that the antibody production was carried out by the same cell clone as produced the initial antibody clone. He proposed a repertoire of randomly diversified cells, each bearing antibodies with a single specificity. Binding of these surface receptors to the antigen was postulated to lead to production of antibodies and replication of the cell. One of the early experiments supporting Burnet's theory was carried out by Nossal and Lederberg [1958]. They showed that single cells harvested from rats immunised with a flagella protein from two different strains of *Salmonella* would make antibodies to one or the other strain, but not to both. This demonstrated that the antibodies produced by each cell have a single specificity. During the 1990s, it became possible to test the essential features of the Burnet model - the idea of Darwinian selection from a large repertoire of diverse antibodies. Winter and colleagues [Winter, 1998] created a large combinatorial antibody library in which

antibody fragments were displayed on the surface of filamentous bacteriophage. The repertoire ( $>10^7$ ) was selected for binding to a wide range of antigens, leading to the isolation of antibody fragments with diverse activities and binding affinities characteristics of primary antibody responses.

Some of the earliest clues to how the receptor diversity is created was presented by Hozumi and Tonegawa [1976]. They fractionated genomic DNA that had been digested by restriction endonuclease BamHI on an agarose gel. mRNA molecules, encoding the constant and variable domain of the antibody, were then analysed for hybridisation with genomic DNA fragments of different sizes. The genomic DNA fragments that hybridised with the mRNA molecules were significantly smaller if the DNA was isolated from mouse plasmacytoma cells (malignant B-cells) instead of cells of 12 to 13 days old mouse embryos. This suggests that the distance between the gene segments encoding the constant and variable domain was reduced in the plasmacytoma and that somatic rearrangement of the antibody genes takes place in B-cells. Similar hybridisation studies in the same laboratory with fragments of cDNA encoding the light chain of the antibody molecule showed that two gene segments (termed V and J) encoding the variable domain also are brought closer together by somatic recombination [Brack et al., 1978]. By cloning and sequencing the hybridising DNA fragments from embryonic DNA and myeloma it was shown that the V is directly linked to the J segments after the recombination [Bernard et al., 1978]. V and J segments have also been identified in heavy chain of the antibody in addition to a third segment called the D segment [Tonegawa, 1983]. One of each V, D and J or V and J segments are put together in the heavy chain or light chain respectively in the rearrangement process. The whole process results in each cell having only one receptor specificity.

Seidman and his colleagues showed that cDNA corresponding to one of the 25 known light chain V segments identified in mice plasmacytomas, hybridised with six EcoRI fragments of genomic DNA from mouse plasmacytoma cells [Seidman et al., 1978]. Cloning and sequencing of the fragments showed that these were closely related V seg-

ments. This suggested that the genome may contain large sets of distinct and related V genes. Some of the later estimates of the number of different antibody gene segments in the genome were mainly based on hybridising cDNA of known segment sequences with mRNA from spleen cells or peripheral blood lymphocytes. Bentley [1984] showed that the about 25 known human germ-line  $V_\kappa$  (see below) genes hybridised with more than 50% of  $\kappa$  chain mRNA from such cells. The total amount of mRNA was estimated by using a DNA probe which hybridises with a constant sequence which is present in all the of  $\kappa$  chain mRNA molecules. From this Bentley concluded that there are about 50 or fewer  $V_\kappa$  in the human genome. The different gene segments have later been more rigorously identified and counted by using the polymer chain reaction (PCR) to amplify the germ line sequences followed by sequencing of the amplified segments [Cook and Tomlinson, 1995]. Knowledge of invariant sequences that surround these gene segments in the germ line, but are deleted in the rearranged antibody sequence, allowed specific amplification of the germ line segments only.

There are two types of light chains in humans,  $\kappa$  and  $\lambda$ ; each of these are assembled from 40 or 30 V segments and 5 or 4 J segments respectively [Janeway et al., 1999]. This gives a combinatorial diversity of 320 ( $= 40 \times 5 + 30 \times 4$ ). In humans the 65 V segments, 27 D segments and 6 J segments of the heavy chains [Janeway et al., 1999] give a possible diversity of 10,530 ( $= 65 \times 27 \times 67$ ). The combinatorial diversity of the heavy and the light chain together, based on the numbers of V, D and J segments, is therefore about  $3.4 \cdot 10^6$  ( $\approx 320 \times 10,530$ ). The expected diversity is even higher due to changes in the residues in the joining regions; different numbers of nucleotides are added to the ends of the segments by two different processes (P or N nucleotides) or trimmed off in the joining event. It has been estimated that this gives a primary antibody diversity of  $10^{14}$  [Janeway et al., 1999] which is larger than the total number ( $10^{11}$  [Rich et al., 2001]) of B cells in the body.

The rearrangement of gene segments in B cells takes place in the bone marrow where they are produced, followed by a deletion or inactivation of B cells specific for self-

antigens. This has been shown with transgenic mice that have high numbers of HEL specific B-cells and also express membrane bound lysozyme in bone marrow cells. The HEL specific B-cells are eliminated in the bone marrow and do not emerge as peripheral cells. In contrast, if high levels of soluble HEL are expressed in such transgenic mice, then the HEL specific B-cells are not deleted but left in a non-responding state (anergy). These cells do not secrete anti-HEL antibodies and show almost no proliferation in response to T-cell stimulation [Goodnow, 1992].

By inspection of alignments of partial sequences of mouse antibody light chains in the 1970s [Weigert et al., 1970] it was proposed that the genes encoding antibody molecules accumulate mutations. This process, known as somatic hypermutation, leads to mutations which are focused in the binding region of the antibodies (as described in more detail later). Griffiths et al. [1984] studied the antibody response to 2-phenyloxazolone through sequencing of mRNA of hybridomas made from different stages of the immune response. Comparison of the hybridoma sequences with the known germ-line sequences showed that somatic mutations had taken place in the second week of the immune response, concomitantly with an increase in antibody affinity. Their results suggested that the affinity maturation of antibodies is driven by somatic mutations. It is now generally accepted that the B cells with increased affinity for the antigen can have a selective advantage over other clones for further cell division and survival.

### 1.2.2 T cells

Some of the earliest evidence of the cell based immunity was reported by Landsteiner and Chase [1942] who showed that delayed hypersensitivity could be transferred by cells but not by serum. Landsteiner transferred cells or supernatants from peritoneal exudates of animals immunised against picryl chloride to non-immunised animals. Only animals receiving cells showed a skin reaction to picryl chloride in oil after 24 hours. Delayed hypersensitivity is a local tissue reaction and is today known to depend on T cells that



have previously been sensitised by the same antigen.

More knowledge on the role of T cells in the immune response came from experiments carried out by Claman et al. [1966]. They transferred thymus cells (T cells) and/or bone marrow (B cells) from donor mice to mice that had been irradiated to remove the recipient's own immune cells. The irradiated mice were then injected with sheep erythrocytes. Cell samples from the spleen of the irradiated mice were later assayed for their ability to release antibodies that could lyse new sheep erythrocytes that were embedded in agar with guinea pig complement. Both B and T cells had to be injected for efficient cell lysis demonstrating the cooperation of T and B cells.

The T cells can be divided into two groups; T-helper cells, which are crucial for the onset of both humoral and cellular immune responses, and cytotoxic T cells which eliminate infected cells. T-helper cells are mainly regulatory and intimately involved in the B-cell and cytotoxic T-cell response. T-helper ( $T_H$ ) cells are divided into the subgroups  $T_H1$  and  $T_H2$  which both differentiate from  $T_H0$ . IL-12 promotes development of  $T_H1$  cells and IL-4 promotes  $T_H2$  cells [Murphy and Reiner, 2002]. The levels of these subgroups are important for regulating the contributions of the humoral and the cellular side of the immune response.  $T_H1$  cells release  $IFN\gamma$  and primarily promote cell-mediated immunity whereas  $T_H2$  cells release interleukins (ILs) as IL-4, IL-5, IL-10 and IL-13 and promote humoral immunity [Akira et al., 2001]. Interaction between cell surface molecules on  $T_H2$  as CD40L and the T-cell receptor (described later) and their ligands on B cells are also important for activation of the B cells. The presence of activated  $T_H1$  or  $T_H2$  cells are in turn dependent on the type of infection and regulated by the cytokines released by cells at the location of the immune response.

In 1982 Allison and his colleagues made a monoclonal antibody (mAb) against T-cell lymphoma [Allison et al., 1982] that recognised a protein present on T cells but absent on B cells. The protein was a dimer and could be separated into two subunits estimated to be about 39,000 Da and 41,000 Da under reducing condition. Shortly there-

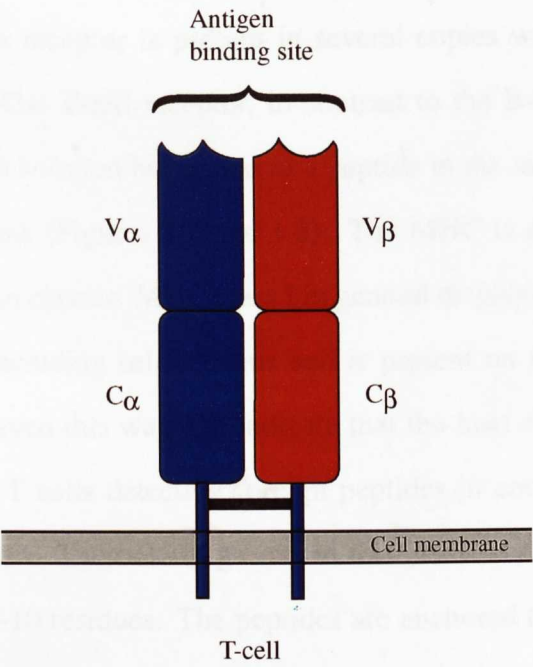


Figure 1.1: *Diagram of the T-cell receptor. The molecule consists of two polypeptide chains  $\alpha$  (blue) and  $\beta$  (red), linked together with a disulphide bonds (black line). The variable regions ( $V_\alpha + V_\beta$ ) form the antigen binding sites.*

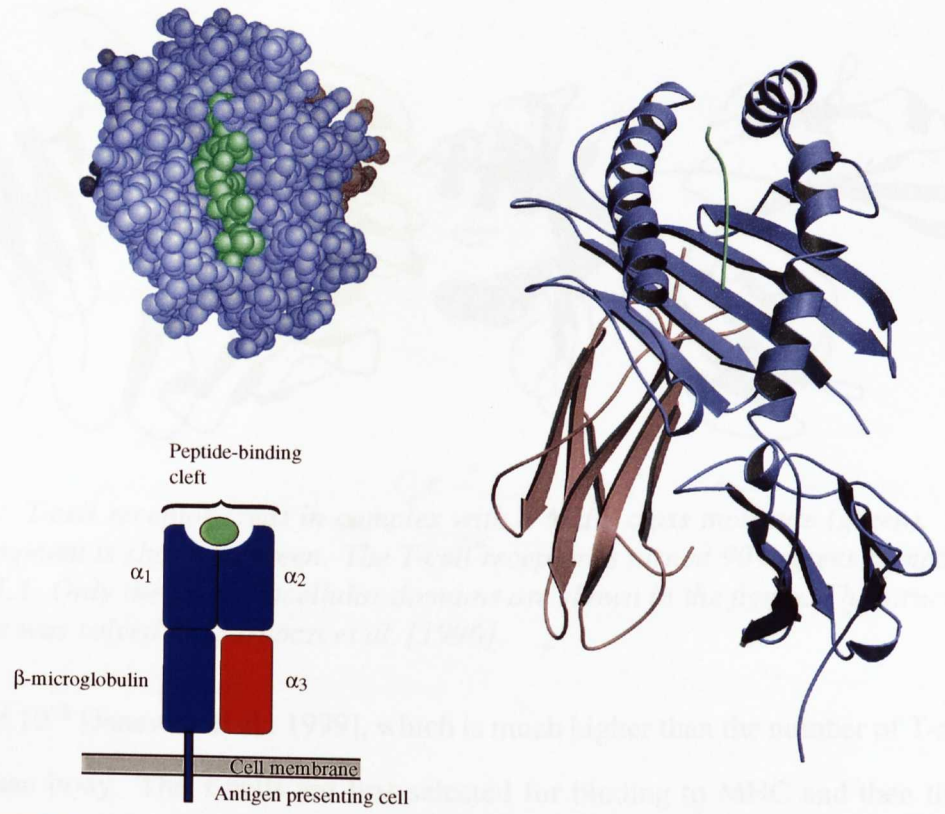
after a mAb was made by Haskins et al. [1983] against a chicken ovalbumin (cOVA) specific T-cell hybridoma. The mAb blocked the production of interleukin 2 (IL-2) by the T-cell hybridoma in response to cOVA and antigen presenting cells but not from other cOVA specific T-cell hybridomas. Furthermore the mAb also blocked binding of the cOVA T-cell hybridoma to antigen presenting cells but only if the antigen presenting cells had been preincubated with cOVA. Not only did this confirm the specific nature of the mAb interaction, but this also suggested that the target of the mAb was a membrane protein. The antibody was found to precipitate a dimeric protein with estimated sizes subunits of about 40-44,000 Da, about the same size as the protein found by Allison and his colleagues. The dimeric protein was proposed by Haskins and her colleagues to be the T-cell receptor which later has been confirmed by other experiments. The T-cell receptor consists of two chains, termed  $\alpha$  and  $\beta$  (Figure 1.1) each with an N-terminal immunoglobulin like variable (V) region and an immunoglobulin like constant (C) region.

The T-cell receptor defines, like the B-cell receptor on the B cells, the antigen speci-

specificities of T cells. The receptor is present in several copies with a single specificity on each T-cell clone. The T-cell receptor, in contrast to the B-cell receptor, does not recognise the antigen in solution but bound as a peptide in the major histocompatibility complex (MHC) proteins (Figures 1.2 and 1.3). The MHC is a cell surface molecule and consists of two main classes. MHC class I in general displays peptides derived from cytoplasmic proteins including self-proteins and is present on most cells in the body. Foreign peptides displayed this way can indicate that the host cell is infected or transformed, and cytotoxic T cells detecting foreign peptides in complex with MHC class I destroy the hosting cell. The oblong groove in this MHC is closed in both ends and displays a peptide of 8-10 residues. The peptides are anchored to the MHC through C-terminal hydrophobic or basic residues and other residues close to the N-terminus. The peptides come from proteins degraded by proteasomes in the cytoplasm. The peptides are transported into endoplasmic reticulum (ER) by TAP-1 and TAP-2 (Transporter associated with antigen processing) and associate with the MHC in the ER. In contrast, MHC class II normally displays peptides of proteins obtained by phagocytosis and is predominantly found on specialised antigen-presenting cells. The peptide in complex with a MHC class II molecule comes from degraded endocytosed proteins and is loaded on the MHC complex in the endosomal compartment. The MHC class II groove is open in both ends and displays peptides of varying length ( $>12$  residues, typically 13-30 residues). The peptides are anchored to the MHC through residues in key positions through the peptide sequence.

Large repertoires of naive T cells with different surface receptor specificities are initially produced. The diversities of their receptors are created by somatic rearrangements of gene segments in a similar way to the B-cell receptor and each T-cell bears a single receptor specificity. The T-cell receptor  $\alpha$  chain is assembled from about 70 D segments and 61 J segments and the  $\beta$  chain is assembled from 52 D segments, 2 D segments and 13 J segments [Janeway et al., 1999]. The combinatorial diversity of both the  $\alpha$  and the  $\beta$  chain is therefore about  $5.8 \cdot 10^6$  ( $\approx 70 \times 61 \times 52 \times 2 \times 13$ ). Additional diversity due to residues added between the gene segments has been suggested to give a possible

MHC class I



MHC class II

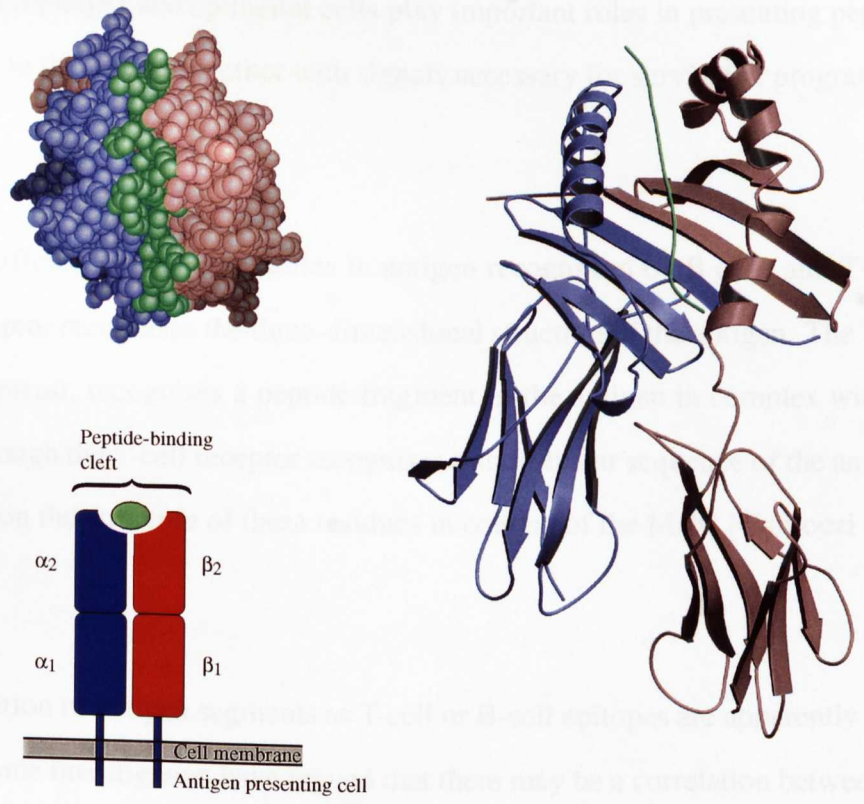


Figure 1.2: Diagrams and cartoons based on the crystallographic structures of MHC class I [Smith et al., 1996] and MHC class II [Murthy and Stern, 1997] molecules. The peptide binding cleft in class MHC class I molecules is formed by the  $\alpha$  chains in contrast to MHC class II in which the cleft is formed by both the  $\alpha$  and the  $\beta$  chain.



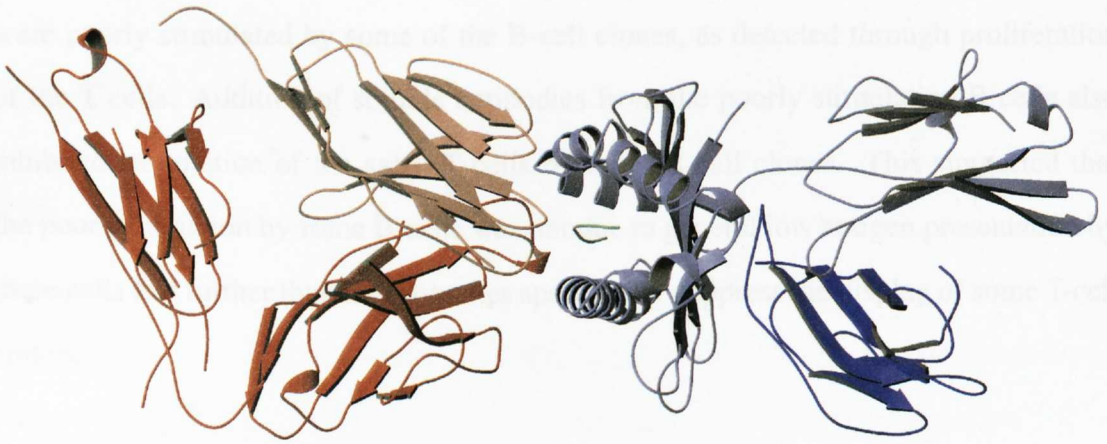


Figure 1.3: *T-cell receptor (red) in complex with a MHC class molecule (green). The antigen fragment is shown in green. The T-cell receptor is turned 90 degrees compared to Figure 1.1. Only the the extracellular domains are shown in the figure. The structure shown here was solved by Garboczi et al. [1996].*

diversity of  $10^{18}$  [Janeway et al., 1999], which is much higher than the number of T-cells in the human body. The T-cells are first selected for binding to MHC and then those specific for self-antigens are deleted. The selection takes place in thymus where dendritic cells, macrophages and epithelial cells play important roles in presenting peptides of self-proteins to the T cells together with signals necessary for survival or programmed cell death.

There are differences and similarities in antigen recognition by B cells and T cells. The B-cell receptor recognises the three-dimensional structure of the antigen. The T-cell receptor, by contrast, recognises a peptide fragment of the antigen in complex with the MHC. But although the T-cell receptor recognises a short linear sequence of the antigen, it is dependent on the structure of these residues in context of the MHC [Garboczi et al., 1996].

The recognition of antigen segments as T-cell or B-cell epitopes are apparently independent. But some investigators have argued that there may be a correlation between the distribution of B-cell and T-cell epitopes in the antigens. Watts and colleagues [Watts and Lanzavecchia, 1993] analysed the ability of different tetanus toxin (tt) specific B-cell clones to activate a panel of tt-specific T-cell clones *in vitro*. Some of the T-cell clones

were poorly stimulated by some of the B-cell clones, as detected through proliferation of the T cells. Addition of soluble antibodies from the poorly stimulating B cells also inhibited stimulation of the same T cells by other B-cell clones. This suggested that the poor stimulation by some B-cells was not due to general low antigen presentation by these cells and further that the antibodies specifically suppress the display of some T-cell epitopes.

Similar studies were carried out by Guermonprez and colleagues [Guermonprez et al., 1999] with antibodies of known affinities. They added hen egg lysozyme (HEL) pre-incubated with different monoclonal antibodies, to antigen presenting cells in the presence of different T cell clones and T-cell activation was detected through IL-2 production by the T-cells. The results from these *in vitro* experiments suggest that some of the monoclonal antibodies suppressed the presentation of specific T-cell epitopes displayed on the surface. It was proposed that some segments of the antigen in the antigen-antibody complex may be protected during intracellular processing of the antigen. Note that the study described above was carried out with relatively high affinity antibodies; low affinity antibodies in primary immune responses may not show such influence on the distribution of T-cell epitopes.

### 1.2.3 The antibody molecule

Antibodies (Figure 1.4) are immunoglobulins (Igs). Edelman [1959] showed that the antibody molecules can be separated into smaller fragments by reduction of disulphide bonds. Two identical heavy chains and two identical light chains are linked together with disulphide bonds. Titani et al. [1965] and Hilschmann and Craig [1965] showed through sequencing of peptides from proteolytically digested antibodies that the antibodies consist of a variable domain with diversity in the amino acid sequence, and a constant domain. Three complementarity determining regions (CDRs) in the variable part of each heavy chain and three CDRs in the variable part of each light chain, com-

prise most of the sequence diversity and form the majority of the antigen binding region (Figure 1.5). The sequence diversity in the CDRs result from the gene rearrangements and somatic hypermutation. CDR1 and CDR2 are encoded in the same V segments. In contrast, CDR3 is encoded by the boundaries between the V and J segments in the light chain and by the boundaries between the V-D segments and the D-J segments in the heavy chain. The CDR3s do therefore contribute to most of the sequence variability in the antibodies. CDR3 of the heavy chain, which also has much higher frequency of N and P nucleotides in CDR3 light chain, contributes most to the sequence variation.

Studies of the DNA encoding the antibodies have shown that the somatic mutations are normally found in a region of 2 kb comprising the V and J gene segments. Introduction of immunoglobulin transgenes with different modifications into B-cell lines have shown that the transcription enhancer elements are important for recruitment of hypermutation to this region [Neuberger et al., 2000]. The molecular basis of the somatic mutations is not fully understood, but sequencing of immunoglobulin genes and comparison with their germline counterparts have revealed several important features as reviewed in Wagner and Neuberger [1996]. The somatic mutations are predominantly found in the variable domains of the antibody and seldom seen in the constant regions (see Figure 1.4). Both framework and CDR residues are mutated although the CDRs tend have the highest mutation rate. Higher mutation rates have been detected in CDR1 compared to CDR2 and CDR3 [Neuberger and Milstein, 1995] in which the diversity is mainly contributed respectively by the different germline V-gene segments and by somatic recombination. The residues that are frequently targets for mutations are termed "hotspots" (in contrast to "cold spots" that are rarely targeted). Most of somatic mutations are single nucleotide substitutions. The substitutions are not completely random but show some preferences for certain nucleotide substitutions and also some codon preferences.

The variable region of the antibody is joined to the constant region which defines the biological functions of the immunoglobulin molecule. The antibodies can be di-

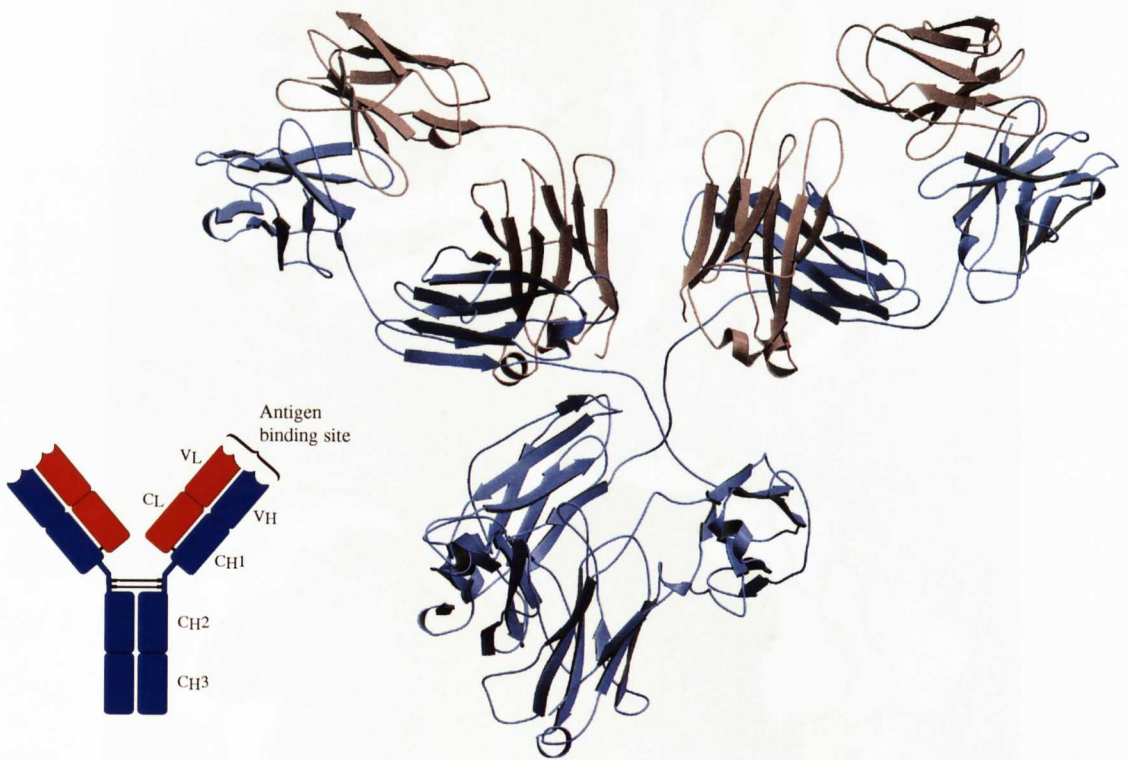


Figure 1.4: Diagram (left) and backbone structure (right) of the IgG molecule. The molecule consists of two heavy chains (H, in blue) and two light chains (L, in red), linked together with disulphide bonds as shown on the left with black lines. The variable regions ( $V_H + V_L$ ) include the CDRs which form the antigen binding sites. All Ig molecules have the same overall shape although they have different number of disulphide bonds and glycosylation patterns. IgM and IgE have four constant domains in the heavy chain ( $C_H$ ) in contrast to three constant domains in the rest of the Ig classes. IgM can form pentamers and IgA can form dimers in association with a protein known as the J (joining) chain. The coordinates in the backbone structure are taken from Harris et al. [1998]



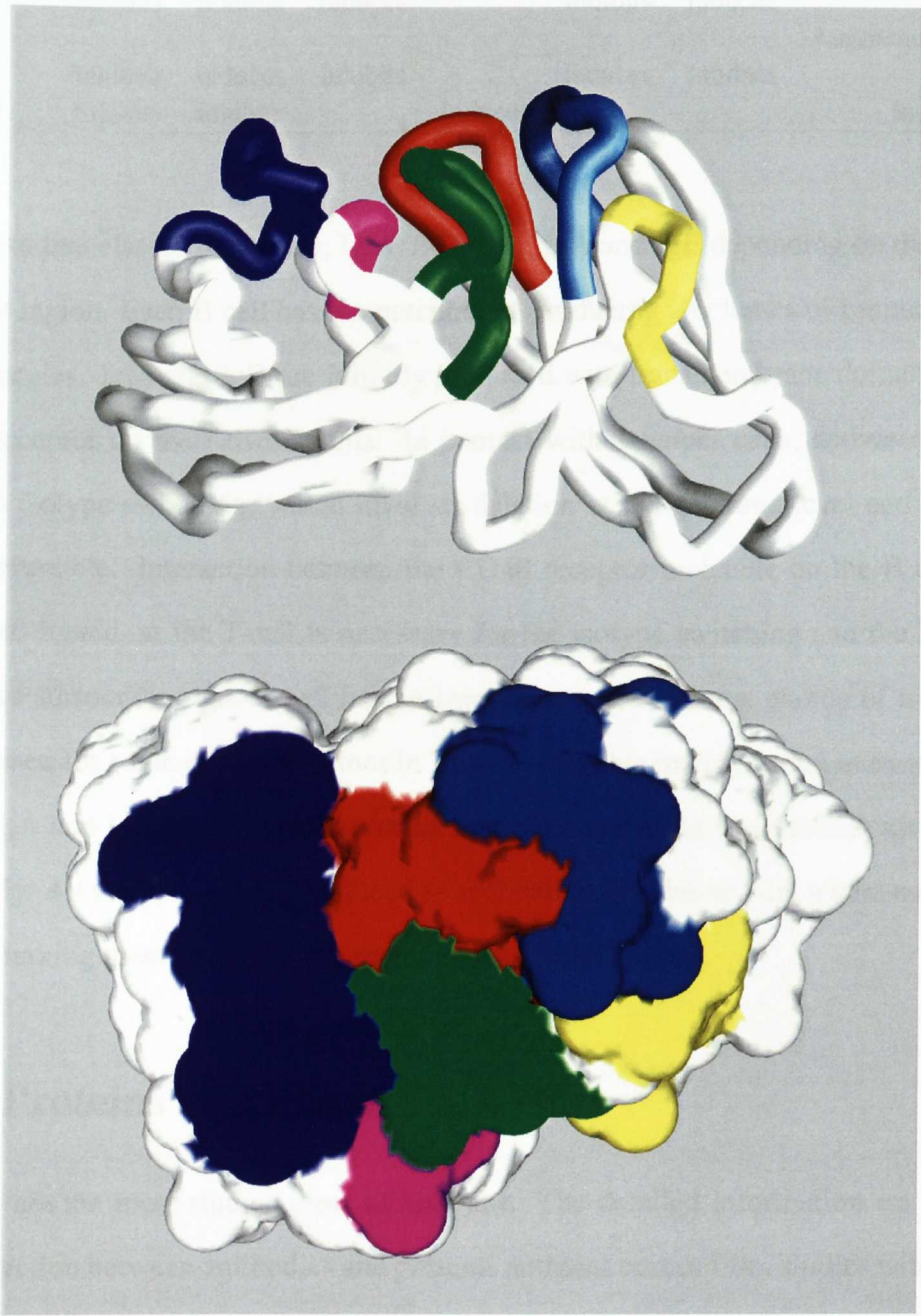


Figure 1.5: The upper image shows the main chain of the variable region of the immunoglobulin molecule seen from the side as in Figure 1.4. The CDRs are shown in different colours. The lower image shows a surface representation of the same molecule, rotated to show the antigen binding region comprised by the CDR regions. The molecule shown here is from Fan et al. [1992].

Table 1.1: *Cytokines known to influence isotype switching in mice. [Janeway et al., 1999].*

Cytokines	IgM	IgG3	IgG1	IgG2b	IgG2a	IgE	IgA
IL-4	Inhibits	Inhibits	Induces		Inhibits	Induces	
IL-5							Augments production
IFN- $\gamma$	Inhibits	Induces	Inhibits		Induces	Inhibits	
TGF- $\beta$	Inhibits	Inhibits		Induces			Induces

vided into five classes (isotypes); IgM, IgD, IgG, IgA and IgE depending on the type of constant region. Each B cell has the potential of producing all classes of immunoglobulin molecules. IgM and IgD are initially produced with transmembrane domains as the B-cell receptor on the naive B cells. In contact with T-helper cells, activated B cells undergo isotype switching, which involves deletion at the genomic level and is therefore irreversible. Interaction between the CD40 receptor molecule on the B cell with the CD40 ligand on the T-cell is necessary for the isotype switching and the levels of cytokines surrounding the B cell has an important impact on the choice of isotype as summarised for some of the cytokines in Table 1.1. IgG is mainly found in extracellular fluids. IgA and IgE are primarily secreted in the gut and close to epithelial surfaces respectively. All isotypes can be produced as secreted molecules or with a transmembrane domain linking them to the plasma membrane as cell receptors.

### 1.3 Proteins as antigens

Proteins are the most studied class of antigens. The detailed information we have on the interaction between antibodies and proteins antigens comes from studies with monoclonal antibodies [Köhler and Milstein, 1975]. Through X-ray crystallography it has been possible to visualise the interaction of antibodies and the antigens on the atomic level.

The first crystallographic structure to be solved of an antibody-antigen complex was the monoclonal antibody D1.3 and hen egg lysozyme (HEL). The structure was solved at 6 Å resolution by Amit and his colleagues in 1985 and improved by the same people

to 2.8 Å resolution the year after [Amit et al., 1986] (Figure 1.6). This showed that D1.3 binds two segments on the surface of HEL which are distant in the linear sequence. The binding surface of the D1.3, which is about 20 by 30 Å, is a relatively flat surface with small irregularities complementary to HEL. The binding energy between D1.3 and HEL is contributed by both van der Waals forces and hydrogen bonds. Two hydrogen bonds are formed between the back bone atoms of the antibody and the backbone atoms of the antigen and four and five hydrogen bonds are formed between the side chain atoms of the antibody and respectively the side chain or backbone atoms of the antigen. 17 antibody residues are in close contact with 16 antigen residues and at least one water molecule is probably also buried in the interface. All six CDRs are in contact with the antigen, although the residues in the CDRs of the heavy chain contribute to most of the interactions with the antigen. This CDR, which contributes most to the sequence variation in antibodies, also forms the physical centre in the interface between D1.3 and HEL. A single residue in the V-region framework of the antibody also contacts the antigen. The interface residues on D1.3 and HEL show no greater movement than 0.6 Å in the complex compared to the free proteins [Mariuzza et al., 1987]. This suggests that the binding is essentially between rigid bodies and that there are only small conformational changes.

Other antibody-antigen structures have been studied and although there are a limited number of solved structures, they have revealed several features which seem to be general to most antibody-antigen interactions [Braden and Poljak, 1995, Lo Conte et al., 1999]. Antibodies bind protein antigens over large sterically and electrostatically complementary surfaces, stabilised by van der Waals forces, hydrogen bonds and in some cases ion pairs. Most of the binding specificity is determined by the CDRs on both heavy and light chain. The CDRs on the heavy chain often dominate in the interaction and of these CDR3 often contributes to most of the binding energy. There are in most cases only small conformational changes in the interface of the antigen and antibody as a consequence of the binding interaction.

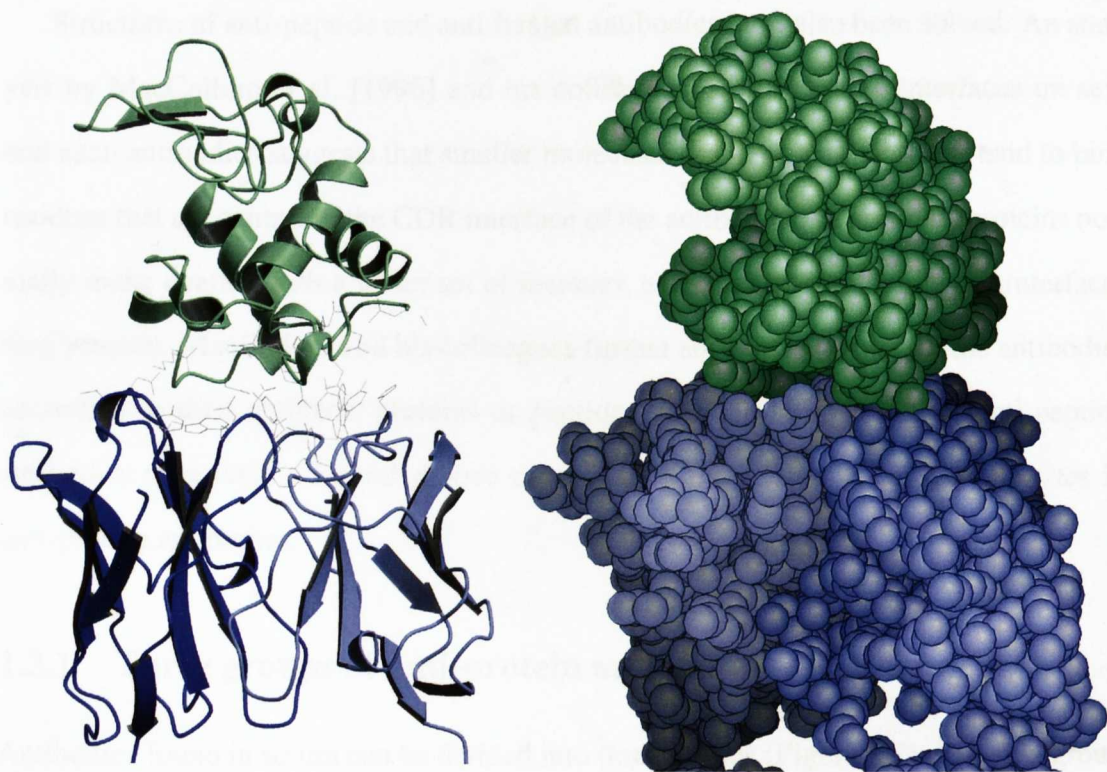


Figure 1.6: The antigen HEL (green) in complex with antibody D1.3 (blue). The complex was solved with the antibody as a Fab fragment ( $V_H$ ,  $V_L$ ,  $C_H1$  and  $C_L$ , see Figure 1.4) and only the variable domain is shown in this figure. The left image represents the backbone structure plus the side chains in the interface. The right image shows an atom representation of the complex. The coordinates are taken from Fischmann et al. [1991].

The average footprint of an antibody is about  $850 \text{ \AA}^2$  (see Table 1 Lo Conte et al. [1999]) and with 15 to 21 residues in contact on the antibody and roughly the same number of residues on the antigen. Binding studies with site directed mutagenesis further suggest that a small number of the interface amino acids contribute to most of the binding energy [Novotny et al., 1989, Smith and Benjamin, 1991a]. These observations are mainly based on antibodies from secondary responses (after somatic hypermutation).

Structures of anti-peptide and anti-hapten antibodies have also been solved. An analysis by MacCallum et al. [1996] and his colleagues of the binding interfaces on several such antibodies suggests that smaller molecules (haptens and peptides) tend to bind residues that are central in the CDR interface of the antibodies. In contrast, proteins normally make contact with a wider set of residues, as expected from the larger interfaces they provide. MacCallum and his colleagues further showed by grouping the antibodies according to their antigens, proteins or peptides, that the binding site of anti-peptide antibodies tend to form a central groove compared to the relatively flat binding sites in anti-protein antibodies.

### 1.3.1 Three groups of anti-protein antibodies

Antibodies found in serum can be divided into three groups (Figure 1.7). The first group only binds to antigen in denatured form and the second group only binds the antigen in native form (respectively groups A and C of Figure 1.7). In this thesis I will refer to these groups as denatured-specific and native-specific antibodies. A third group of antibodies (group B of Figure 1.7) are able to bind both native and denatured forms of the antigen. I will refer to this group as cross-reactive antibodies. These antibodies probably bind different segments of the polypeptide chain that are brought together on the surface of the folded protein, but are also able to bind the separate segments with detectable affinity.

Lando and his colleagues demonstrated the existence of these three groups of antibodies in sera. They immunised rabbit, sheep and goat with sperm whale myoglobin

(Mb). Antibodies from the animal sera were affinity purified on cyanogen bromide (CNBr) cleaved polypeptide fragments of Mb [Lando and Reichlin, 1982a] which are assumed to be linear forms of the antigen. Binding assays with the eluted antibodies were then carried out in solution where labelled antigen or antigen fragments were added to the solution followed by addition of secondary antibodies to precipitate the antibody-antigen complexes. The results showed that  $10^4$ - $10^6$  more intact Mb protein than CNBr fragments of the protein (by weight) was needed to compete with labelled CNBr fragments of the protein for binding to the antibodies. The huge excess of protein needed for competition shows that a fraction of the affinity purified antibodies had much higher affinity for the linear form than for the folded protein. These antibodies represent denatured-specific antibodies.

A fraction of the antibodies that were affinity purified on the CNBr peptides were also shown to bind labelled native Mb in solution [Lando et al., 1982b]. These antibodies were cross-reactive antibodies (group B) as they bound both denatured and native protein. The antibodies that were affinity purified on the CNBr peptides therefore comprised both cross-reactive and denatured-specific antibodies. Lando and his colleagues also found that sera that had been depleted of all antibodies that bound the CNBr fragments contained antibodies that bound native Mb. These native-specific antibodies (group C) counted for 30-40% of the total amount of Mb specific antibodies.

The amount of antibodies detected in the three groups depends on the methods used to analyse the sera as the determination of which antibodies bind to native and denatured forms of an antigen depends on the sensitivity of the methods. Different methods usually operate with different affinity thresholds and can be expected to detect varying amounts of antibodies in the three antibody groups.



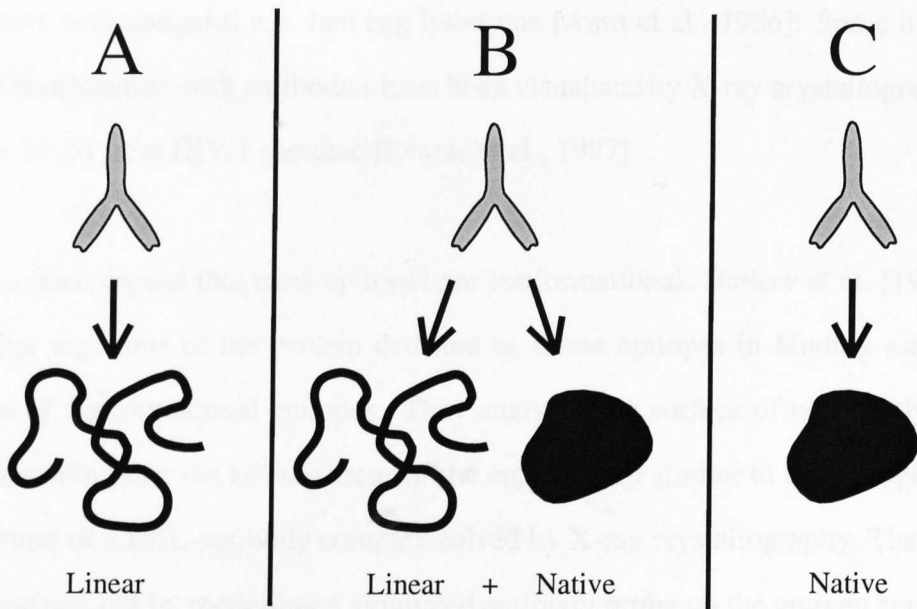


Figure 1.7: *The three groups of anti-protein antibodies in sera. Group A binds only denatured protein and group C binds only native protein. Antibodies from group B bind both native protein and denatured protein. Group A, B and C are here described as denatured-specific, cross-reactive and native-specific antibodies respectively.*

### 1.3.2 Conformational and linear epitopes

The region of the protein bound by an antibody is described as an epitope, antibody determinant or antibody combining site [Jerne, 1960]. Jerne defined epitopes as elements of the native protein which can be bound by antibodies. Elements that only are available for binding when the protein is denatured were defined as cryptotopes.

More commonly used classifications are topographic (conformational or discontinuous) and sequential (linear) epitopes. An antigen surface that is bound by an antibody and comprises segments which are distant in the linear antigen sequence is defined as a topographic or conformational [Sela, 1969a, Sela et al., 1969b] epitope. The contact residues on the antigen are brought together on the antigen surface by the folding of the protein. Whether the antibody is native-specific or cross-reactive depends on the segments ability to bind the antibody individually or together. By contrast, in sequential epitopes the binding to the antibodies is only dependent on the sequence and not on the higher ordered structures in the protein [Sela, 1969a, Sela et al., 1969b]. The existence of conformational epitopes have been confirmed in several crystal structures of antibodies

in complex with antigens, e.g. hen egg lysozyme [Amit et al., 1986]. Some linear epitopes in combination with antibodies have been visualised by X-ray crystallography, e.g. residues 36-46 from HIV-1 protease [Lescar et al., 1997].

It has been argued that most epitopes are conformational. Barlow et al. [1986] proposed that segments of the protein detected as linear epitopes in binding assays, are elements of conformational epitopes. They analysed the surface of hen egg lysozyme (HEL) assuming that the surface areas of the epitopes are similar to the epitope seen in the structure of a HEL-antibody complex solved by X-ray crystallography. Their analysis was carried out by modelling a simulated antibody probe on the antigen surface and examining the antigen sequence included in the interface. Taking each atom on the antigen surface in turn they checked the fraction of neighbouring residues that are within 10 Å radius. This showed that the footprint of an antibody of this size on the antigen surface most likely include antigen residues that are more than 10 residues away in the linear sequence.

### **1.3.3 Immunogenicity and antigenicity.**

Immunogenicity is the potential of a substance (antigen) to evoke an immune response leading to humoral or cellular products that specifically recognise the substance. Antigenicity is the ability to be recognised by the antibody produced in a humoral immune response. Thus a free hapten is not immunogenic, whereas presented as a haptened protein is both immunogenic and antigenic.

### **1.3.4 Distribution of epitopes on the antigen surface**

Antigens can originate from transplanted tissue, infectious organisms, allergens or self-proteins in autoimmune diseases. Proteins are the most diverse group of antigens the immune system encounters and the possible surface structures on proteins are almost unlimited. The distribution of epitopes on the antigen surface can be examined in two steps. First, what parts of an antigen are antigenic? Second, what is the relative dis-



tribution of antibodies to the different antigenic regions of a protein? Much data has been produced by investigators to answer the first question, but very little regarding the second question.

### **Which parts of an antigen are antigenic?**

The investigation of antigenicity of different parts of antigens has been a long process with several pitfalls, mainly due to the methods used. The first model antigen with known crystallographic structure was sperm whale myoglobin [Crumpton and Wilkinson, 1965]. Crumpton and Wilkinson showed some of the proteolytic fragments of Mb could partly inhibit the precipitation of Mb by antiserum, which indicated that these regions were antigenic.

Atassi and his colleagues later claimed to have solved the precise and entire antigenic structure of sperm whale myoglobin [Atassi, 1975] and HEL [Atassi and Lee, 1978]. They described five peptides of six to eight amino acids and three peptides of five to six amino acids that accounted for the complete antigenic anatomy of Mb and HEL respectively. They reported that all IgG specific for Mb and HEL in sera of animals immunised with these proteins can be absorbed by these peptides. However, later results from other laboratories are inconsistent with these claims. For example Lando and his colleagues [Lando et al., 1982b] found, as described earlier, that 30-40% of anti-myoglobin antibodies do not bind any of the three major cyanogen bromide fragments of Mb that together account for the entire protein sequence. Others have also showed that several monoclonal antibodies to Mb do not bind any of these fragments [Berzofsky et al., 1982].

Benjamin and colleagues [Benjamin et al., 1984] evaluated data collected through several studies of antibody responses to the model antigens myoglobin, lysozyme, cytochrome c and serum albumin. The methodologies differed. For example the epitopes on myoglobin were identified by mapping studies of serum antibodies with peptides and of monoclonal antibodies by homologues of Mb (method described later), whereas the

epitopes for serum albumin were identified by competition assays with monoclonal antibodies and the mapping of serum antibodies with fragments of serum albumin. Taken together the collection of epitope data suggests a more complex picture of antigenic surfaces than earlier described. Benjamin and his colleagues concluded; *"The surface of a protein antigen consists of a complex array of overlapping potential antigenic determinants; in aggregate these approach a continuum. Most determinants depend upon the conformational integrity of the native molecule"*. This has gained a general acceptance in the later literature.

### **The relative distribution of antibodies on the protein surface**

Benjamin and his colleagues demonstrate in their reappraisal [Benjamin et al., 1984] that most if not all the parts of an antigen surface will be recognised by antibodies if a sufficiently large number of humoral immune responses are considered. Although they mention that some parts of the antigens seem to attract more antibodies than others they did not evaluate the relative distribution of antigenicity over the antigen surfaces. A large body of earlier literature on B-cell epitopes describe the presence of dominant epitopes (e.g. [Morgan et al., 1992] or [Worobec et al., 1985]). Dominant epitopes have also been linked to the intrinsic features of the antigens. Westhof and colleagues [Westhof et al., 1984] showed that there is a positive correlation between the identified dominant epitopes in tobacco mosaic virus protein, myoglobin and lysozyme with the backbone mobility of the proteins. However, the presence of dominant epitopes seems surprising from an evolutionary point of view as the failure to recognise any part of the antigen represents a loophole for pathogens. It would therefore be expected that the constant battle against rapidly evolving pathogens should direct the immune system to respond efficiently and therefore equally well to all protein surfaces.

Although the presence of dominant epitopes is mentioned multiple times in the literature, the data supporting existence of dominant epitopes on native protein are weak. Thus although the studies of monoclonal antibodies in complex with antigens have al-

lowed us to see epitopes in molecular detail, they do not provide information about the relative surface distribution of the antibodies. Serum antibodies include all this information. But the composition of antigen specific antibodies in serum is very complex. This makes it difficult to analyse their epitope distribution. One means has been the use of synthetic peptides to analyse the binding characteristic of antibodies. But although peptides may simplify the problem of epitope mapping they will be ignored by native-specific antibodies.

Assays aimed at mapping both native-specific and cross-reactive antibodies do therefore require native protein. Protein homologues have been used in such assays [Urbanski and Margoliash, 1977, Jemmerson and Margoliash, 1979] as they have small variation surface residues. Differential binding of serum antibodies to different homologues may allow identification of some of the conformational epitopes. The model proteins used in these studies were typically proteins with highly conserved sequence from different species such as mitochondrial cytochrome c and bird egg lysozymes.

Again this method has severe limitations, as there are a limited number of native homologues. Weber and his colleagues [Weber et al., 1992] used a method they called Homologous Scanning Mutagenesis to create more variants as illustrated with human growth hormone (hGH). Short segments of hGH were replaced with the corresponding segments of homologues and the new chimaeric proteins were then analysed for the ability to bind serum antibodies raised against the wild type hGH in mice. The results suggested that five of the 19 substituted regions dominated the antibody response in mice. But the method has its own limitations. For example variants of chimaeric protein with different mutations in the same segment showed very different binding activity to serum antibodies. The mutant hGH with segment 54-74 transplanted from human prolactin showed significant reduction in binding for only one of five sera whereas the mutant hGH with segment 57-73 transplanted from porcine growth hormone showed significant reduction in binding for all five sera. This suggests that the differential binding of serum antibodies to the wild type and the mutants was dependent on the specific sequence and

not only on the surface location of the substitutions.

There was, to my knowledge, no method that can be used to systematically identify the relative distribution of epitopes of serum antibodies on the three-dimensional protein surfaces. This was the motivation behind the epitope mapping assay developed during this work.

### **$\beta$ -Lactamase as model antigen**

The use of antigens with homologues in the immunised species would be expected to complicate the relationship between epitope distribution and intrinsic properties of the antigen as the distribution could be strongly influenced by regulation mechanisms of the antibody response to the host antigen [Jemmerson and Margoliash, 1979] as discussed later in more detail. A bacterial protein,  $\beta$ -lactamase, with no expected homologues in the immunised species was therefore chosen as a model antigen.

$\beta$ -Lactamase (Figure 1.8) provides bacteria with resistance to  $\beta$ -lactam antibiotics such as penicillin and ampicillin. The gene encoding the protein was originally found in the transposable element Tn3 [Heffron et al., 1979]. The protein is a monomer and consists of both  $\beta$ -sheets and  $\alpha$ -helixes and has a molecular weight of 30,000 Da. It has two cysteines which form an internal disulphide bond. The surface area is 11,200 Å<sup>2</sup> which corresponds to roughly the sum of the binding areas of 13 mutually exclusive antibodies, assuming an binding interface of 850 Å<sup>2</sup> (as described earlier). The structure of the protein is known and it is well expressed in bacteria.  $\beta$ -Lactamase has an enzyme activity which can be detected with chromogenic substrates. This should allow verification of the presence of native protein as the enzyme activity requires the native conformation of the protein.

Different strategies for mapping the epitopes of native-specific and cross-reactive antibodies were considered. The chosen strategy was to make a variety of cysteine mu-

transfers in the surface of  $\beta$ -lactamase and to couple them to a solid surface through a bifunctional chemical cross linker. The orientation of the antigen on the solid surface, defined by the cysteine position, would be expected to mark specific regions of the surface from binding to antibodies.

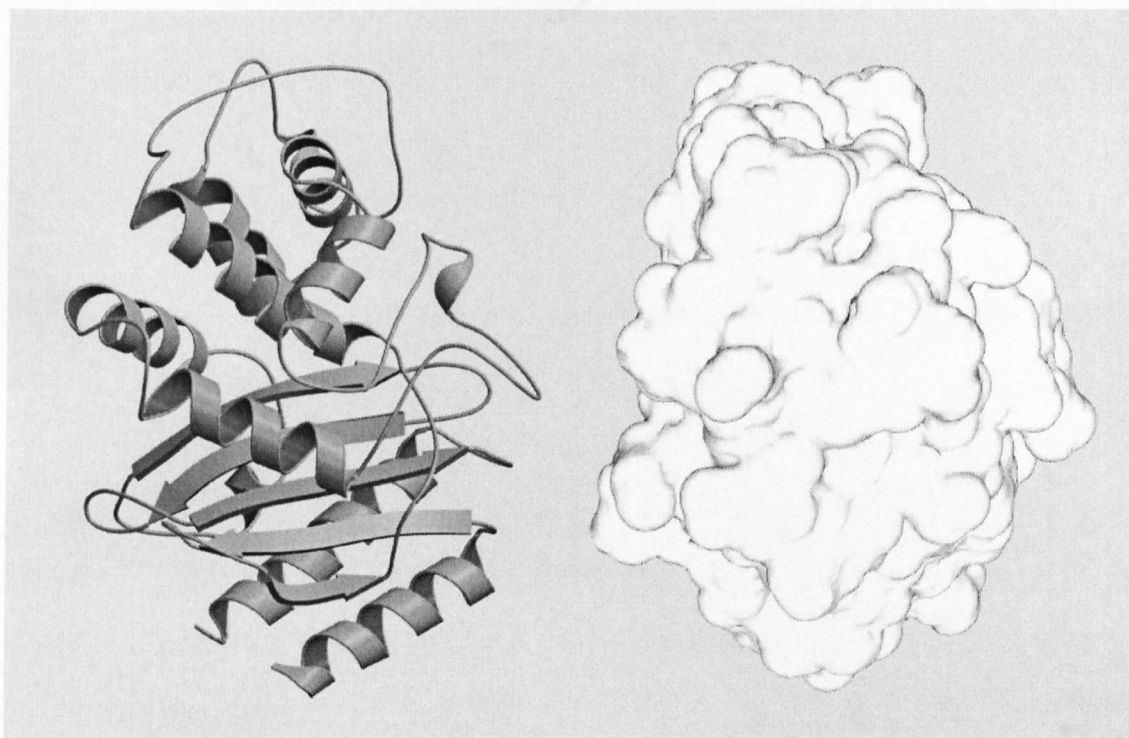


Figure 1.8: The left image shows the secondary structure of  $\beta$ -lactamase. The protein is a monomer with a molecular weight of 30,000 Da. The right image shows a surface representation of the protein. The surface is defined by the centre of a spherical probe, with a radius 1.4 Å, rolled over the protein atoms. The surface area is 11 200 Å<sup>2</sup>. The structure shown here was solved by Jelsch et al. [1993].

tations in the surface of  $\beta$ -lactamase and to couple these to a solid surface through a bifunctional chemical cross-linker. The orientation of the antigen on the solid surface, defined by the cysteine position, would be expected to mask specific regions of the surface from binding to antibodies.

# Chapter 2

## Mapping monoclonal antibodies

### 2.1 Introduction

The key to map the topological epitopes was to mask the different surface regions of the antigen by coupling to a solid surface as shown in Figure 2.1. A cysteine introduced on the antigen surface by mutagenesis was chemically attached to a biotin-linker (biotin molecule with a linker) which in turn was captured by streptavidin on a solid surface. The biotin-linker together with the streptavidin molecule masks the surrounding antigen surface. This was expected to completely exclude binding of antibodies in the proximity of the cysteine.

The antigenicity of the whole antigen surface can be analysed by systematically placing the cysteine residue, the tethering point, in different surface locations and thereby creating an array of the antigen in different orientations. The distribution of cysteine residues should be chosen so that any antibody would be excluded from binding by at least one cysteine position. The areas of the surface that were masked from antibody binding by the biotinylation and capture on the solid surface, will here be referred to as "cysteine target regions" (CTRs). Monoclonal antibodies were first used to evaluate the method as they offer more defined conditions than sera.

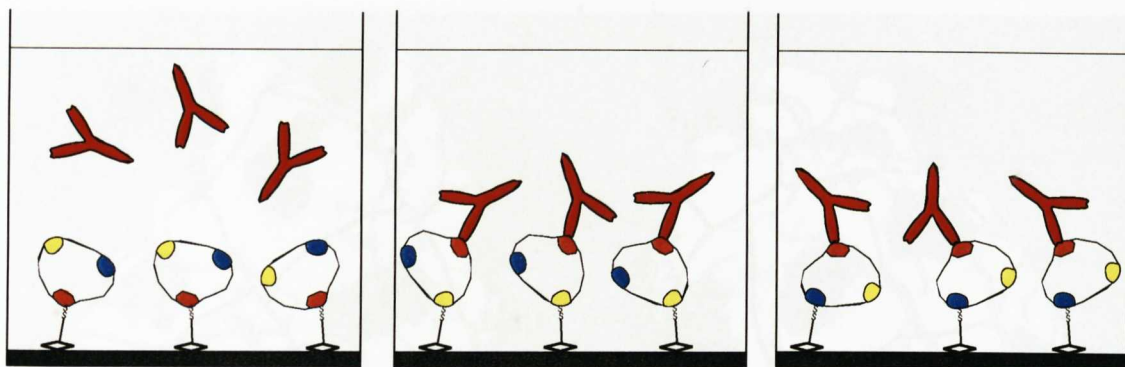


Figure 2.1: Mapping of monoclonal antibodies on the antigen surface. Different regions (only three in this example) of the antigen surface are blocked by a biotin-linker which is tethering the antigen to streptavidin coated ELISA plate wells through a cysteine on the antigen surface (see text). Tethering through the binding region (red) does not allow binding of the antibody. In contrast, presentation of the antigen in different orientations allows binding of the same antibody.

## 2.2 Results

### 2.2.1 Cysteine mutations and biotinylation of $\beta$ -lactamase

The surface of the model antigen  $\beta$ -lactamase was systematically divided into 23 evenly distributed regions (see Figure 2.2). (The total antigen surface of  $11,200 \text{ \AA}^2$  corresponds to about 13 non-overlapping antibody-antigen interfaces of  $850 \text{ \AA}^2$ .) A free cysteine was placed in the middle of each region by mutagenesis. The positions for the cysteine mutations were chosen by eye using the computer software GRASP [Nicholls et al., 1991] to visualise the three-dimensional protein surface. Mutations were made among surface residues such that the sulphydryl groups would be expected to be surface accessible. The distance between neighbouring cysteine positions was about  $20 \text{ \AA}$ . One mutant with a single surface cysteine was then prepared for each of the chosen cysteine positions. The mutants were K34C, N52C, K55C, E63C, Q90C, N100C, E110C, T114C, T140C, K146C, N154C, N175C, E197C, L201C, A202C, K215C, A227C, E240C, D254C, Q269C, T271C, E281C, K288C and 308C (with a cysteine added on the C-terminus after the peptide affinity tags). The use of the C-terminal cysteine mutant 308C is described in the next chapter. All mutants were expressed in *E. coli* although at different levels (see Table 2.1). The panel of cysteine mutants was biotinylated with a sulphydryl specific biotin-linker (see Figure 2.3) which had a  $29 \text{ \AA}$  spacer arm that linked the biotin molecule to the



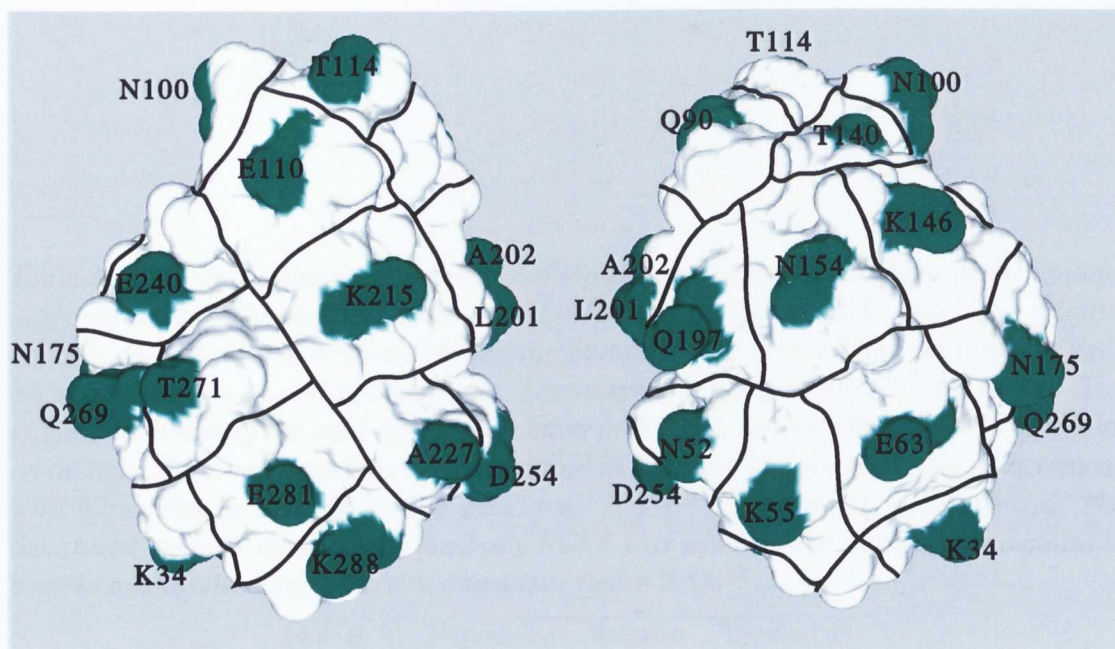


Figure 2.2: The epitope mapping regions defined by the 23 cysteine mutations. The 23 surface residues mutated into cysteines are in green. The black lines illustrates how the antigen surface is divided into different regions.

protein through a disulphide bond. An important condition for the current mapping assay to work was specific biotinylation on the cysteines. Mass spectrography results indicate that only one biotin-linker was attached to each protein molecule (representative data are shown in Figure 2.4) as expected for the thiol-specific biotinylation. The mutants had the expected additional mass of about 450 Da for the addition of the biotin-linker and no evidence of further additions of biotin.

### 2.2.2 State of the $\beta$ -lactamase mutants

The state of the  $\beta$ -lactamase mutants were analysed to ensure that all the mutants included native protein. The presence of denatured protein among the mutants was also analysed. The importance of this population will become more apparent during mapping of serum antibodies in the next chapter.

Table 2.1: The table shows an overview of the  $\beta$ -lactamase mutants used in the epitope mapping assay. The mutants were expressed in the periplasm of *E. coli*. The relative enzyme activities were measured when the mutants were biotinylated and immobilised on a streptavidin coated ELISA plate. The enzyme activity of 308C is set to 1.0. The enzyme activities of the wild type  $\beta$ -lactamase and 308C, representing the wild type with an added C-terminal cysteine, were compared in solution.  $k_{cat}$  for wild type  $\beta$ -lactamase with Nitrocefin in PBS pH 7.4 at 22°C was  $164\text{ s}^{-1}$  and  $K_m$  was  $4.58 \cdot 10^{-5}\text{ M}$ . The denatured specific-monoclonal antibody P64.1 was used to detect traces of denatured protein among the biotinylated mutants (see figure 2.5).

Mutant	Expression per litre of culture [mg]	Relative activity	Binding to monoclonal antibody P64.1
Wild type	12.35	1.034*	
K34C	1.16	1.034	+
N52C	2.57	0.926	-
K55C	1.57	0.997	+
E63C	3.57	0.946	-
Q90C	1.79	0.550	-
N100C	2.18	0.980	-
E110C	1.47	1.031	Poorly
T114C	4.42	1.119	-
T140C	8.47	0.981	-
K146C	3.27	1.002	+
N154C	7.81	0.971	-
N175C	0.27	0.869	+
E197C	3.96	0.990	-
L201C	13.75	1.007	-
A202C	8.50	0.995	-
K215C	8.19	1.023	+
A227C	16.38	1.151	-
E240C	7.00	0.758	-
D254C	12.53	1.003	-
Q269C	5.48	1.065	-
T271C	2.93	0.698	-
E281C	10.69	0.610	-
K288C	2.21	0.864	-
308C	10.94	1.000	-

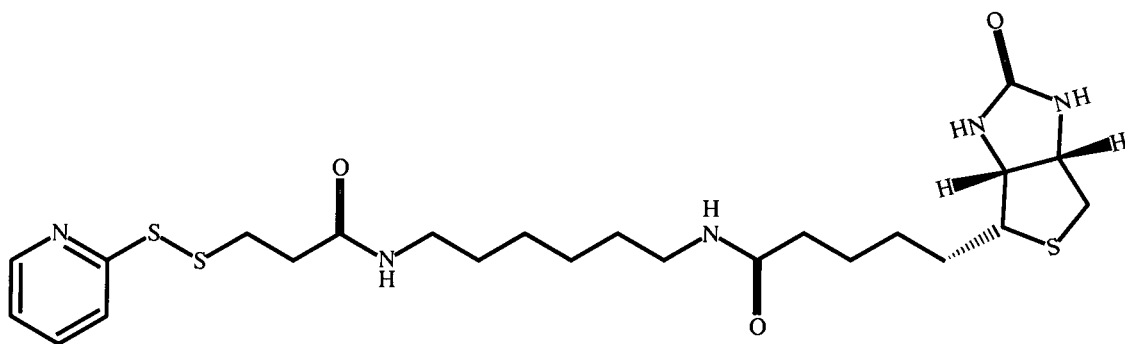


Figure 2.3: The biotin-linker HPDP used in the epitope mapping assay. The linker has a pyridyl-disulphide group (left) coupled through a spacer arm (29 Å) to a biotin molecule. The pyridyl leaving-group will exchange with the thiol group on the surface of the protein. The result is a biotin molecule with attached through the linker to the protein by a disulphide bond. The molecular weight of the HPDP is 539.8 Da.

### Analysed through enzyme activity

Enzyme activity can be used to detect native  $\beta$ -lactamase as it requires the native conformation of the protein. The relative enzyme activities of the 23  $\beta$ -lactamase mutants linked to streptavidin plates are shown in Figure 2.5a. All the mutants had enzyme activity although some variation was observed. This shows that there was some native protein immobilised on the plates, and that the amounts of native protein were similar between the different mutants.

### Analysed with monoclonal antibodies

Small amounts of denatured  $\beta$ -lactamase were expected to be difficult to detect through changes in enzyme activity and a denatured-specific monoclonal (P64.1) was therefore used as a probe. The 23 cysteine mutants were biotinylated and immobilised on a streptavidin coated ELISA plate. Binding of the monoclonal antibody was detected with labelled anti-mouse antibodies (Figure 2.5b). The signals detected indicated the presence of some denatured protein for 5 of the 23 mutants. The two  $\beta$ -lactamase mutants, K34C and N175C, that most efficiently bound P64.1 were poorly expressed in *E. coli* (Table 2.1).  $\beta$ -Lactamase mutant 308C was not recognised by the same monoclonal antibody (data not shown).

The enzyme activity  $k_{cat}/K_m$  for wild type  $\beta$ -lactamase type in solution with Ni-

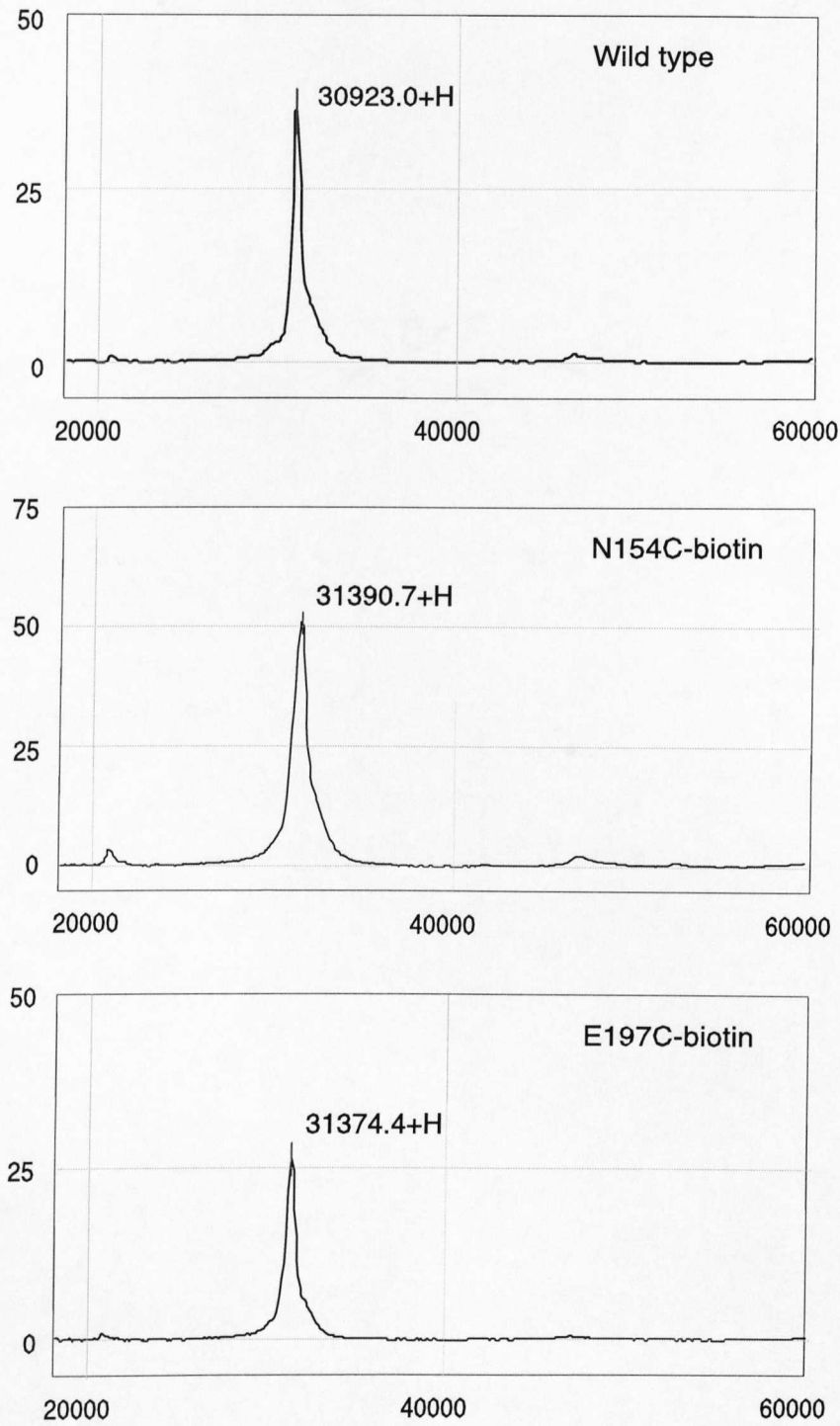


Figure 2.4: Mass spectrography results for the biotinylated  $\beta$ -lactamase mutants. Expected mass for the wild type (with tags) is 30948.99 Da. The data recorded for the mutants suggest homogeneous populations of protein with only one biotin-linker per protein molecule.

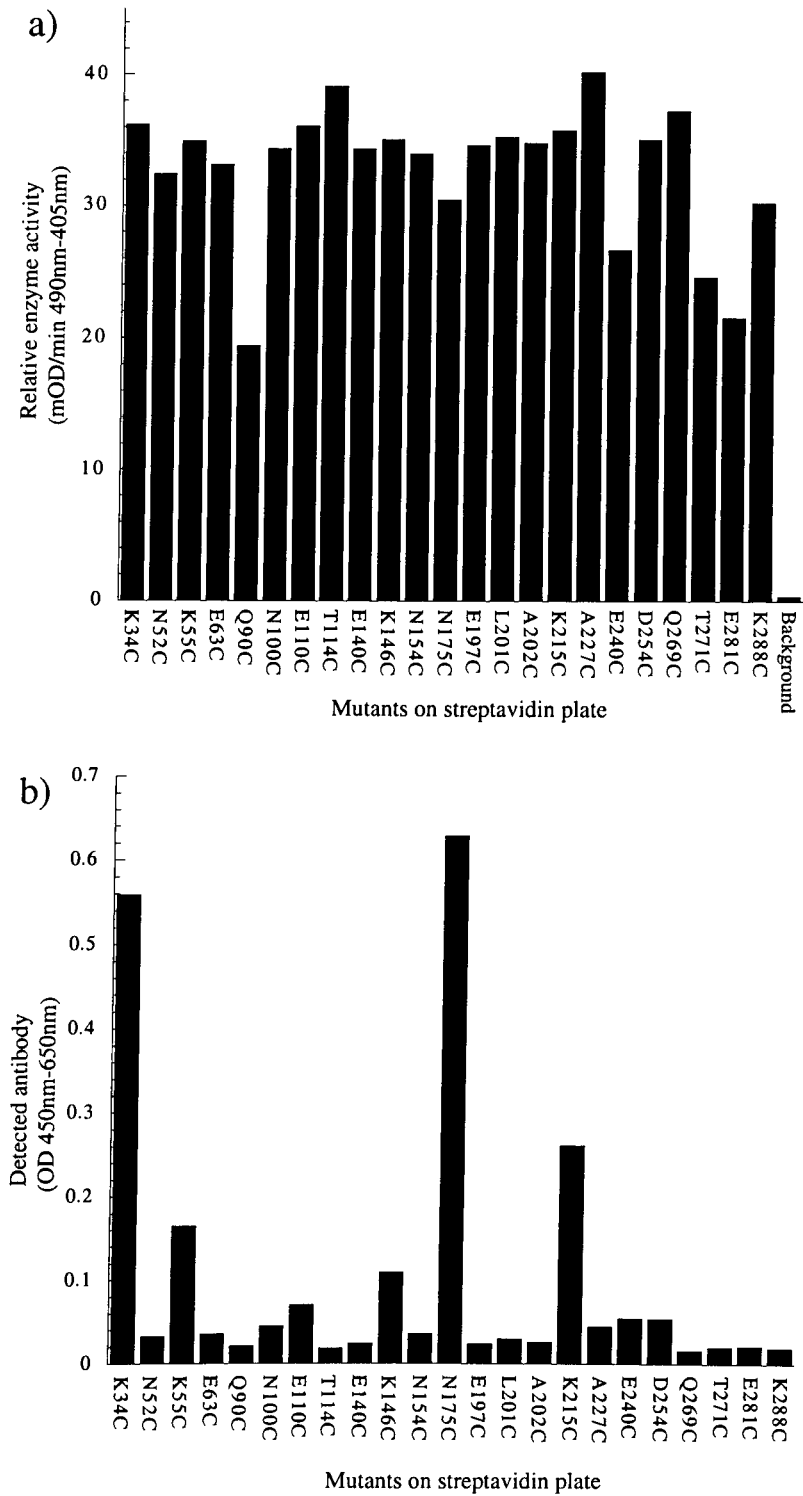


Figure 2.5: State of  $\beta$ -lactamase mutants. a) Biotinylated  $\beta$ -lactamase mutants were immobilised in ELISA wells coated with streptavidin. The relative enzyme activities of  $\beta$ -lactamase mutants were measured with the chromogenic substrate Nitrocefin. 308C has an enzyme activity very close to L201C and A202C (separate experiment). A denatured-specific monoclonal antibody (P64.1) was used to evaluate if any of the  $\beta$ -lactamase mutants contained fractions of denatured protein. The biotinylated  $\beta$ -lactamase mutants were immobilised on streptavidin coated ELISA plates. The background is 0.02.

trocefim (in PBS pH 7.4 at 22°C) was  $3.58 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$  where  $K_m$  was  $4.58 \cdot 10^{-5} \text{ M}$ . The  $k_{cat}$  of the biotinylated 308C under the same conditions was  $159 \text{ s}^{-1}$  compared to  $164 \text{ s}^{-1}$  for the wild type. (Osuna et al. [1995] reported a ratio  $k_{cat}/K_m$  of  $4.23.58 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$  for the wild type  $\beta$ -lactamase.)

### 2.2.3 Production of monoclonal antibodies

Monoclonal antibodies were produced against wild type  $\beta$ -lactamase. This work was done under close supervision and with help from Tone Varaas at the Central Laboratory, The Norwegian Radium Hospital, Oslo. The monoclonal antibodies were made with spleen cells from one mouse that had been immunised with  $\beta$ -lactamase. The mouse was initially injected twice subcutaneously with  $\beta$ -lactamase in Freund's complete adjuvant (FCA) followed by a subcutaneous injection with  $\beta$ -lactamase in Freund's incomplete adjuvant (FIA), all with three weeks intervals. The mouse was then injected intravenously each of the four days prior to the cell fusion with  $\beta$ -lactamase in PBS. The media from the small cultures of fused cells were assayed for antibodies that bound native or denatured  $\beta$ -lactamase. The native antigen was displayed as biotinylated 308C captured on streptavidin coated plates. The denatured antigen was  $\beta$ -lactamase coated directly on onto ELISA plates that were subsequently dried. The absence of native protein on these plates was verified by adding the chromogenic substrate Nitrocefim. The presence of protein was verified with anti-serum. The screening of media from 960 cultures gave about 450 cultures that tested positive on native protein and 70 cultures that tested positive on denatured antigen. Some of these media aliquots did also give signal on both native and denatured antigen. Cells from 72 cultures were sub-cloned. 11 of the sub-cloned cells were then grown in larger cultures (Table 2.2). This included two native-specific, seven cross-reactive and two denatured-specific antibodies. IgGs from five of these cultures were purified on protein G. The yields ranged from 5-10 mg for 200 ml cultures.

The monoclonal antibodies' abilities to immobilise  $\beta$ -lactamase from solution were

Table 2.2: *Monoclonal antibodies displayed on ELISA plates were used to immobilise  $\beta$ -lactamase added in solution. The enzyme activity of  $\beta$ -lactamase was used to detect bound protein. None of the monoclonal antibodies block the enzyme activity (data not shown). \*The monoclonal antibodies were also assayed for their ability to bind biotinylated  $\beta$ -lactamase captured on streptavidin coated ELISA plates (data not shown). The denaturation was done by reducing an internal disulphide bond and carboxymethylate the two cysteines.*

	Detected $\beta$ -lactamase [mOD/min]	Binds native $\beta$ -lactamase*	Binds denatured $\beta$ -lactamase*
P50.2	0.66	+	+
P51.1	15.84	+	Poorly
P58.3	2.56	+	+
P59.1	9.84	+	-
P64.1	0.36	-	+
P65.2	0.36	-	+
P66.1	0.67	+	+
P67.2	0.40	+	+
P68.1	0.36	Poorly	+
P69.1	27.78	+	+
P70.1	16.25	+	+
Background	0.29		

also examined. The monoclonal antibodies were captured on ELISA plates coated with anti-mouse antibodies.  $\beta$ -Lactamase was then added in solution and allowed to bind to the monoclonal antibodies. The bound  $\beta$ -lactamase was detected with the chromogenic substrate Nitrocefin. The results are shown in Table 2.2. There were large differences in the antibodies ability to bind  $\beta$ -lactamase from solution. This probably reflected the relative affinity of the antibodies for the antigen.

2.2.4 Mapping of monoclonal antibodies

The nine subcloned monoclonal antibodies that bound the native protein were mapped with the method described in the introduction of this chapter. In an enzyme-linked immunosorbent assay (ELISA) the monoclonal antibodies were analysed for their ability to bind the 23 biotinylated mutants immobilised on streptavidin coated ELISA plates. Bound monoclonal antibodies were detected with secondary antibodies conjugated to an enzyme (horseradish peroxidase). The results are shown in Figures 2.6, 2.7 and 2.8.

Most of the monoclonal antibodies were excluded by more than one of the cysteine target regions (CTRs). This supports the idea that the panel of mutants was suitable for detecting binding regions of serum antibodies specific for any parts of the antigen surface. The biotin-linker's ability to exclude binding seems to depend on the antibody affinity for the antigen. Monoclonal antibodies which poorly immobilised  $\beta$ -lactamase from solution (e.g. P68.1, see Table 2.2) were partly excluded by biotin attachment points over larger parts of the surface. The most precise determination of binding site was achieved for the antibodies that efficiently immobilised  $\beta$ -lactamase from solution as seen with P69.1 and P70.1.

The binding regions of the monoclonal antibodies are shown on the three-dimensional surface in Figures 2.9 and 2.10. The CTRs with most dramatic decrease in binding of each monoclonal antibody cluster together on the antigen surface and are presumably included within the antibodies' footprints. Other CTRs with intermediate signals are close on the antigen surface.

## 2.3 Discussion

Mapping topographic epitopes requires folded protein. All the mutants had enzyme activity and were recognised by one or both the native-specific antibodies P51.1 and P59.1. This shows that all the mutants contained folded protein. Furthermore most of the mutants contained no denatured protein as shown by a denatured-specific antibody. Five of the mutants did contain some denatured protein that was recognised by the denatured-specific antibody. But these five mutants had similar enzyme activities to the rest of the mutants (and as indicated above all bound equally well to at least one of the native-specific antibodies). This suggests that the fraction of denatured protein in the five mutants must be small. The uniform levels of folded protein for each mutant allow direct comparison of the mutants binding monoclonal antibodies.

The resolution of the epitope mapping is dictated by the size of the excluded antigen



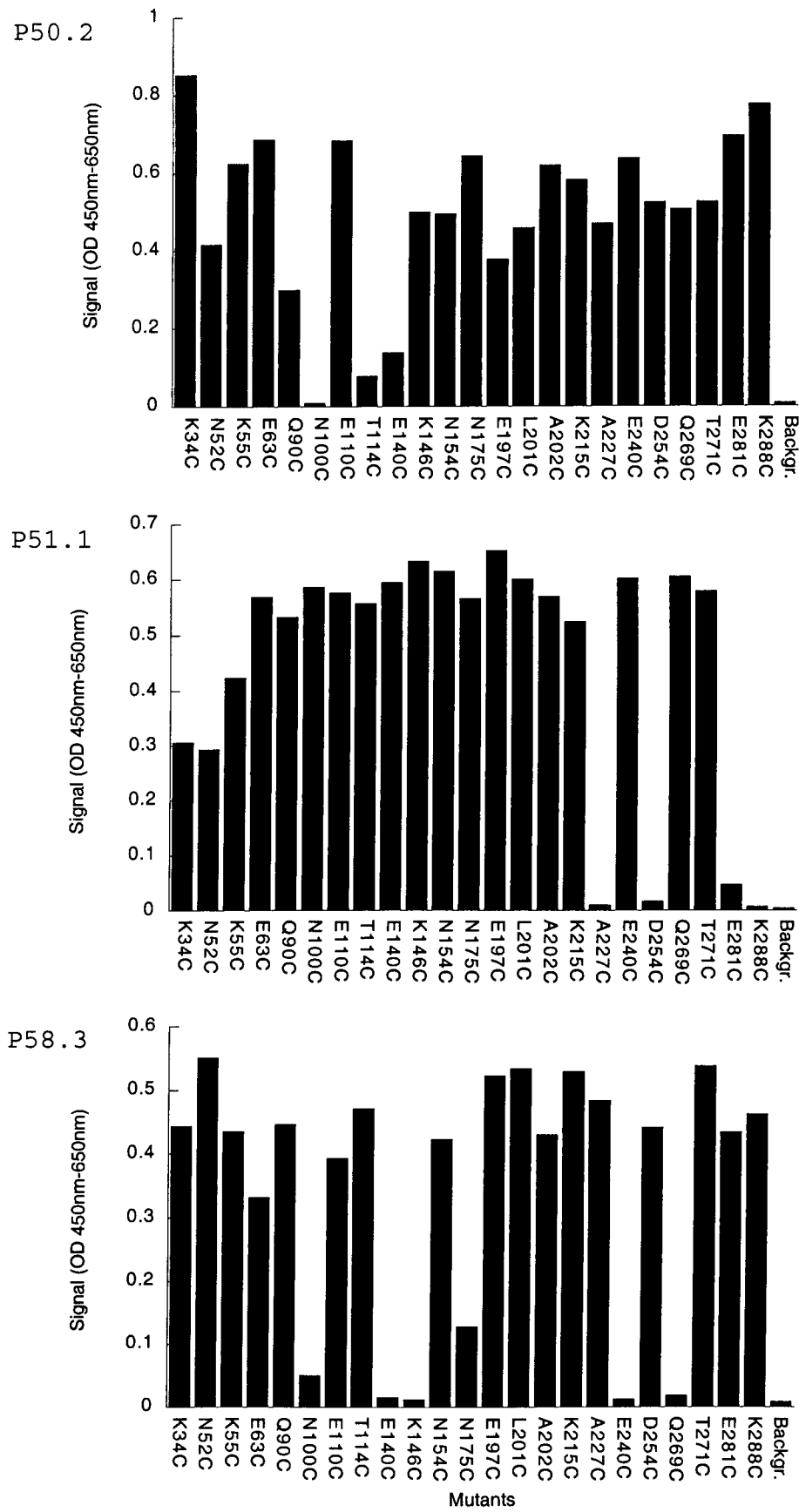


Figure 2.6: Monoclonal antibodies P50.2, P51.1 and P58.3 were assayed for their ability to bind the 23 different  $\beta$ -lactamase mutants. The biotinylated mutants were captured on streptavidin coated ELISA plates. The cysteine mutations with low signal map close together on the surface for each monoclonal antibody.

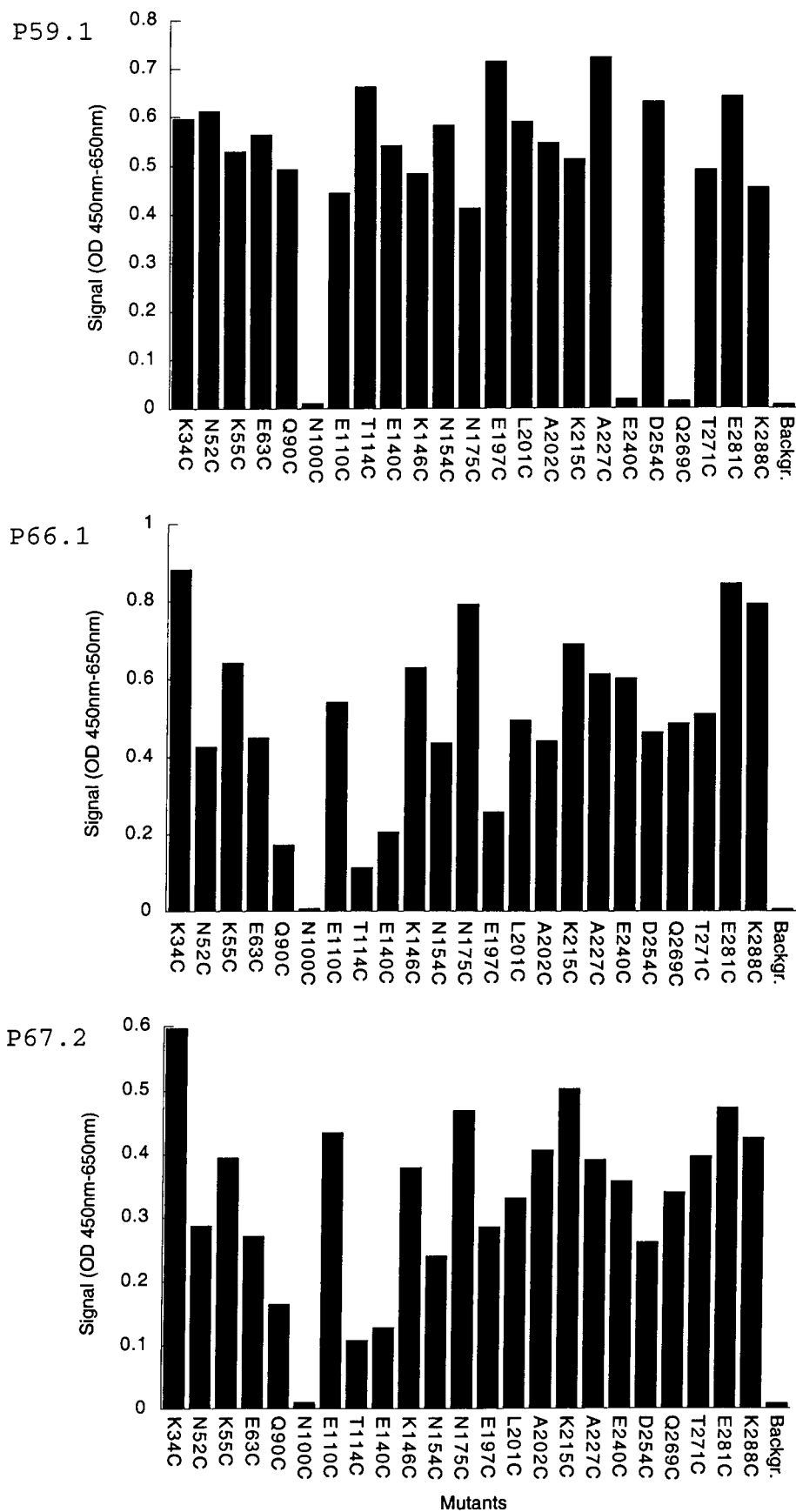


Figure 2.7: Monoclonal antibodies P59.1, P66.1 and P67.2 were assayed for their ability to bind the 23 different  $\beta$ -lactamase mutants. The biotinylated mutants were captured on streptavidin coated ELISA plates. The cysteine mutations with low signal map close together on the surface for each monoclonal antibody.

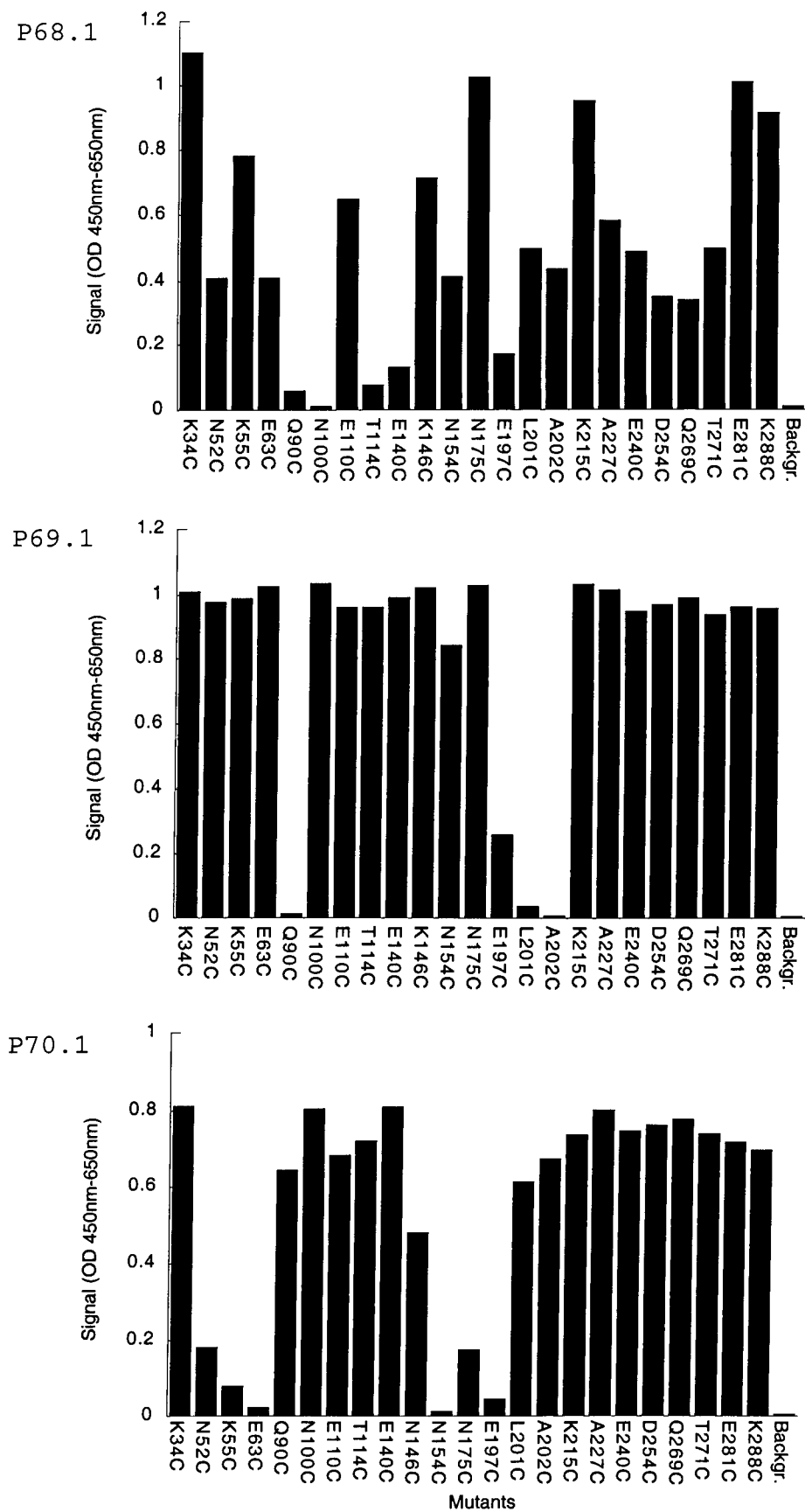


Figure 2.8: Monoclonal antibodies P68.1, P69.1 and P70.1 were assayed for their ability to bind the 23 different  $\beta$ -lactamase mutants. The biotinylated mutants were captured on streptavidin coated ELISA plates. The cysteine mutations with low signal map close together on the surface for each monoclonal antibody.

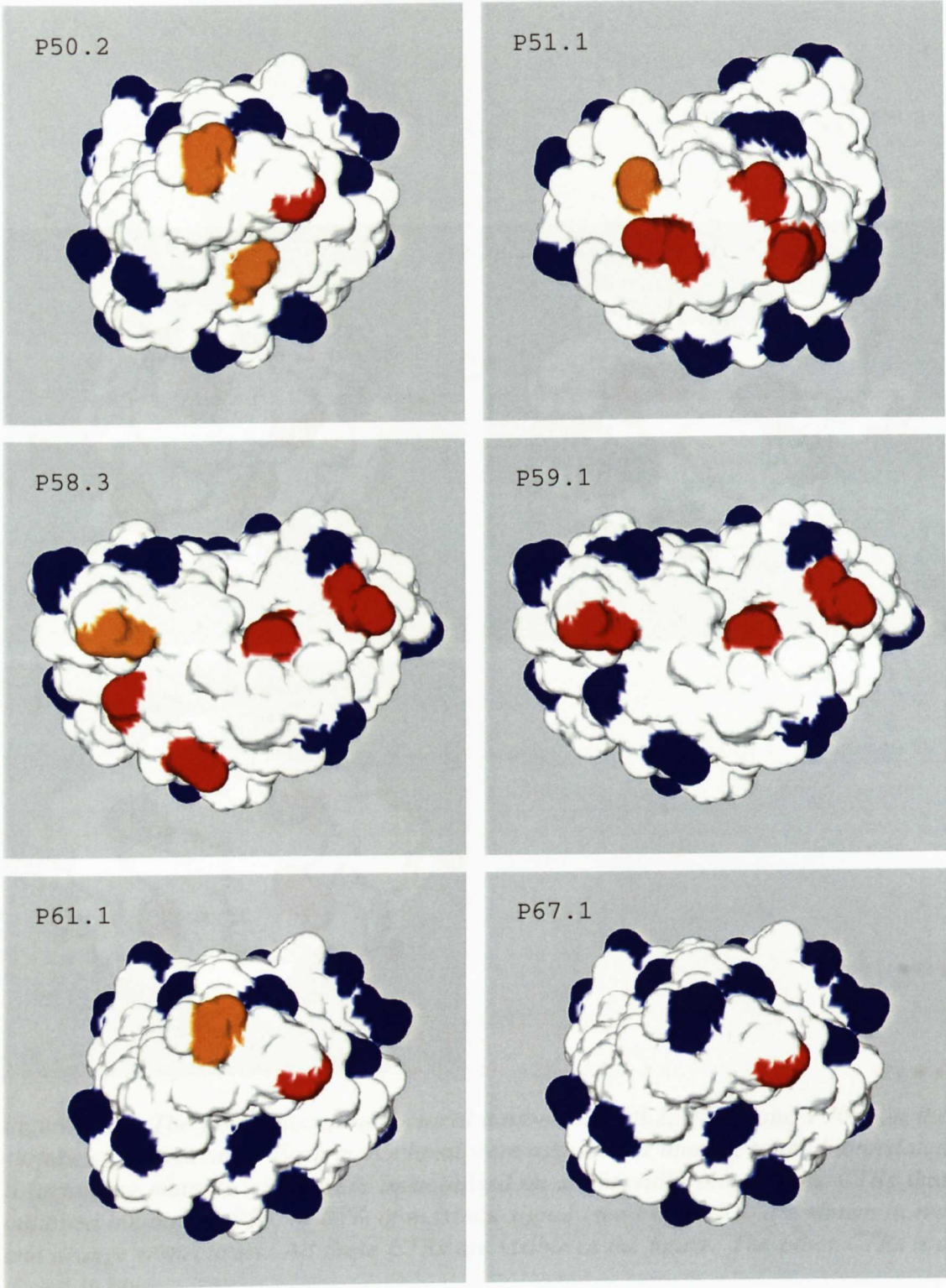


Figure 2.9: The footprints of monoclonal antibodies P50.2, P51.1, P583, P59.1, P59.1, P61.1 and P67.1 on the surface of  $\beta$ -lactamase. Each monoclonal were assayed for binding to the biotinylated  $\beta$ -lactamase mutants which were immobilised on streptavidin plates. The CTRs that inhibited binding by 95% or 85% of maximal signal (see Figures 2.6 and 2.7) are shown in red and orange respectively. All these CTRs are visible in the figure. The other CTRs are shown in blue.

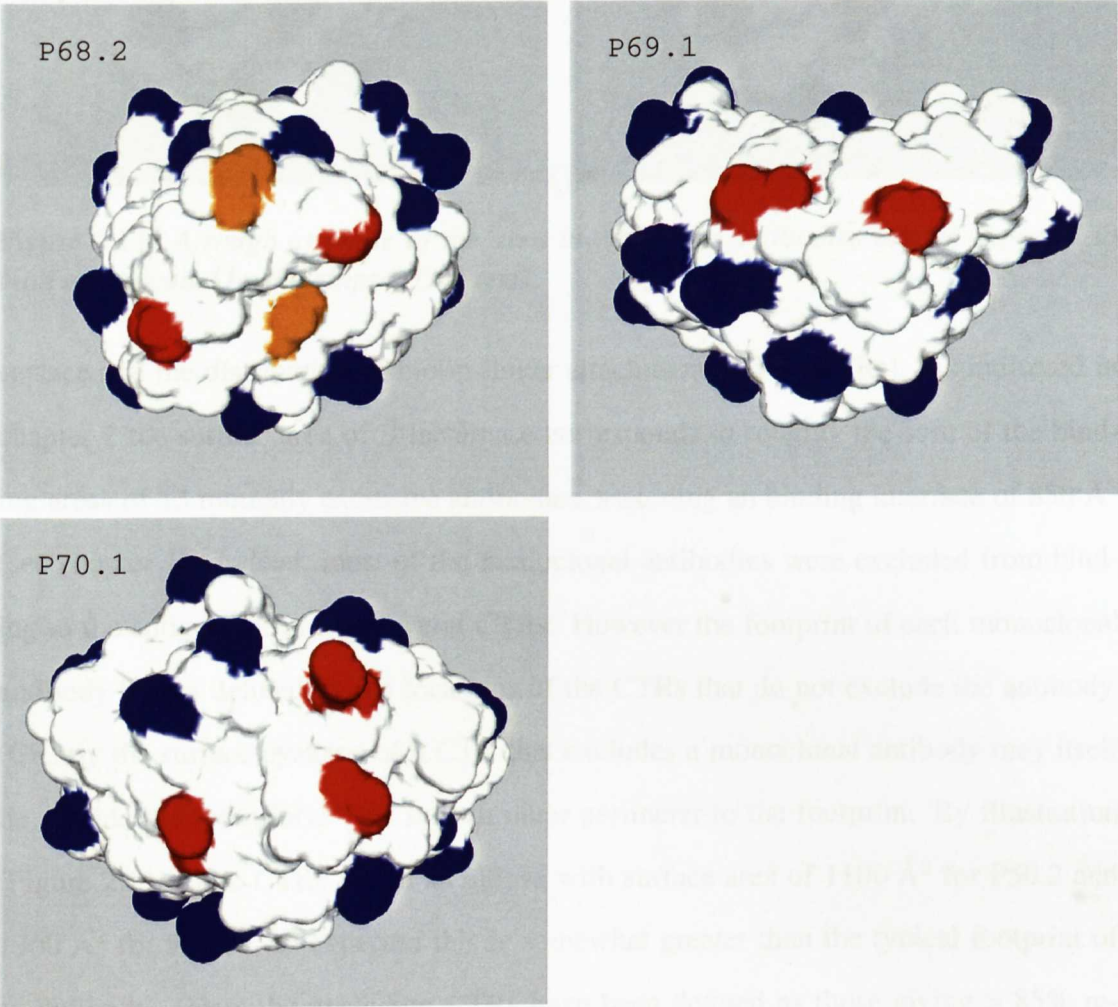


Figure 2.10: The footprints of monoclonal antibodies P68.2, P69.1 and P70.1 on the surface of  $\beta$ -lactamase. Each monoclonal were assayed for binding to the biotinylated  $\beta$ -lactamase mutants which were immobilised on streptavidin plates. The CTRs that inhibited binding by 95% or 85% of maximal signal (see Figure 2.8) are shown in red and orange respectively. All these CTRs are visible in the figure. The other CTRs are shown in blue.



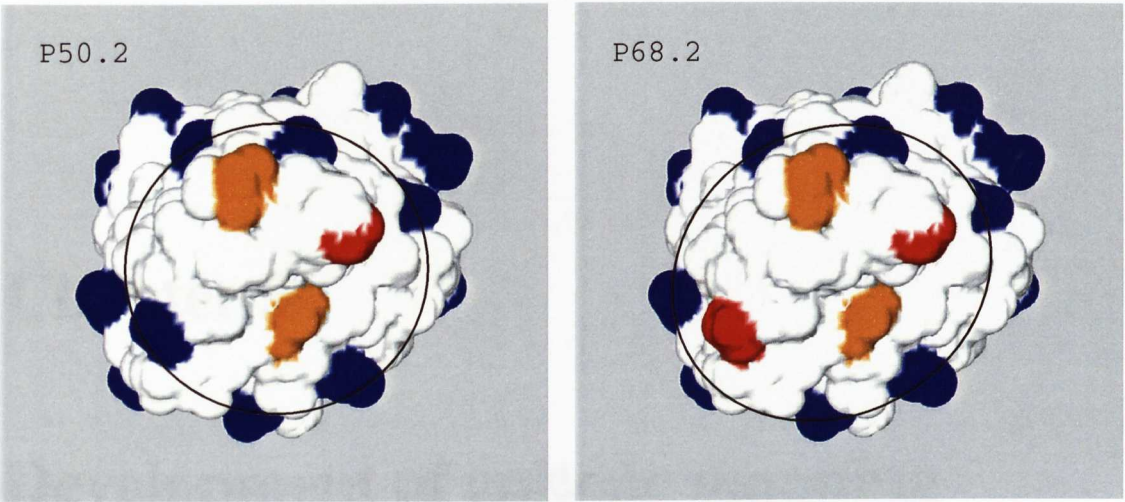


Figure 2.11: A rough estimate of the area in which the antibodies can be expected to bind as indicated by the ellipses (see text).

surface and the distribution of biotin-linker attachment points (CTRs). As indicated in chapter 1 the surface area of  $\beta$ -lactamase corresponds to roughly the sum of the binding areas of 13 mutually exclusive antibodies, assuming an binding interface of  $850 \text{ \AA}^2$  (see chapter 1). Indeed, most of the monoclonal antibodies were excluded from binding to the antigen surface by several CTRs. However the footprint of each monoclonal antibody is best defined by the locations of the CTRs that do not exclude the antibody. (Clearly the surface cysteine of a CTR that excludes a monoclonal antibody may itself lie outside the footprint). This sets an outer perimeter to the footprint. By illustration (Figure 2.11) these CTRs fall in an ellipse with surface area of  $1100 \text{ \AA}^2$  for P50.2 and  $1300 \text{ \AA}^2$  for P68.2. As expected this is somewhat greater than the typical footprint of an antibody. (Here the excluding CTRs have been defined as those giving  $> 85\%$  reduction in signal.) The resolution of the method therefore appears capable of defining the epitope within a surface area that is about 50% larger than a typical antibody footprint. The resolution of the method could presumably be improved by using more CTRs.

# Chapter 3

## Development of epitope mapping method for serum antibodies

### 3.1 Introduction

The method presented in chapter 2 applies to monoclonal antibodies. The challenge was here to map the epitope distribution of serum antibodies. The principles of the solution are illustrated in Figure 3.1. The method is based on exclusion of antibodies from predefined surface regions as shown for monoclonal antibodies (chapter 2) but was modified to subtract serum antibodies to all surface regions except the biotinylated regions (CTRs). The remaining antibodies to the chosen CTRs could then be quantified.

The same panel of 23 cysteine mutants was used for the mapping of serum antibodies as for the monoclonal antibodies. The biotinylated mutants were in this case captured on streptavidin coated magnetic beads. Beads with the different mutants were then mixed with separate aliquots of the sera. The serum antibodies that bound the antigen were then pulled out of solution together with the beads. Multiple cycles of depletion were necessary, as described in methods on page 132. The antibodies remaining after 10 cycles of depletion and specific for the masked CTR, were then detected using unmasked antigen.  $\beta$ -Lactamase 308C was in general used as unmasked antigen. The aliquots were also tested with the same masked antigen as used for depletion. In the event of complete

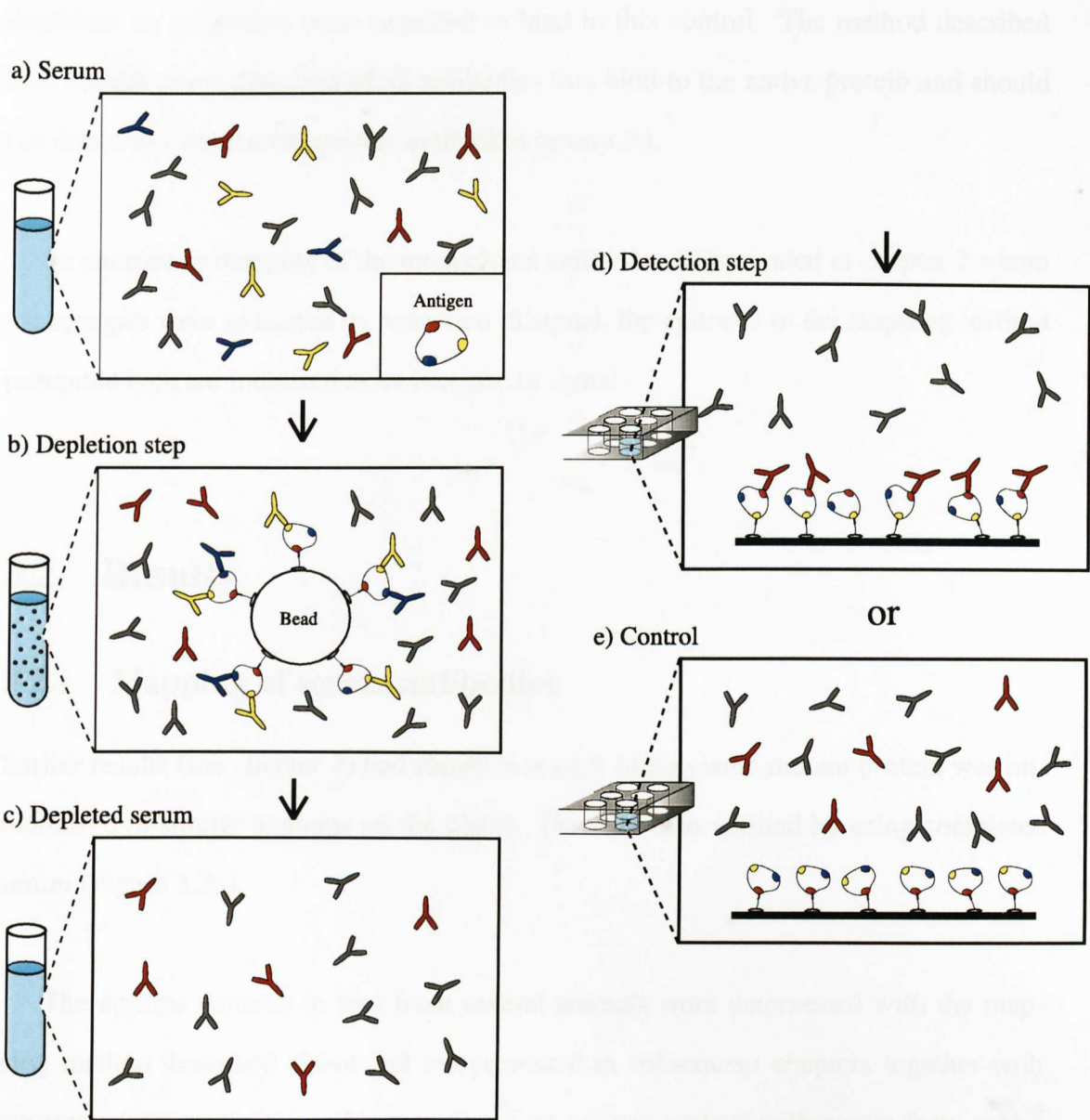


Figure 3.1: The principles in the method used to map the relative distribution of serum antibodies on the protein surface. a) The binding regions (only three in this example) are coloured in red, blue and yellow and the corresponding antibodies in the same colours. The grey antibodies are not specific for this antigen. The goal is to detect antibodies to each of the regions separately. b) The antigen is biotinylated on a cysteine mutated into the region of interest as shown here with the red region. The biotinylated protein is captured on magnetic streptavidin beads which are then mixed with serum. All the antigen specific serum antibodies can bind the antigen surface, except the antibodies that are specific for the region masked by the biotin-linker. c) The red antibodies are the only antibodies specific for native protein left in the solution after pulling out the beads. d) The amount of antibodies specific for the red region is detected on a solid phase. Antigen which is biotinylated on the surface, at a point far away from the region of interest, are captured on streptavidin coated ELISA plates. This leaves the region of interest exposed to binding of the red antibodies. Secondary antibodies conjugated to an enzyme are used to determine the amount of red antibodies. e) Control: Depleted serum from (c) is tested for ability to bind antigen when the red region is masked by the biotin-linker.



depletion no antibodies were expected to bind in this control. The method described here should allow detection of all antibodies that bind to the native protein and should not detect any denatured-specific antibodies (group A).

In contrast to mapping of the monoclonal antibodies as presented in chapter 2 where the epitopes were indicated by reduction in signal, the epitopes in the mapping method presented here are indicated as an increase in signal.

## 3.2 Results

### 3.2.1 Mapping of serum antibodies

Earlier results (see chapter 2) had shown that each biotinylated mutant protein was immobilised in similar amounts on the plates. This was also verified by using completed serum (Figure 3.2a).

The epitope patterns in sera from several animals were determined with the mapping method described above and are presented in subsequent chapters together with the immunisation regimes. An example of an epitope pattern with serum from rabbit 1 is shown in Figure 3.2b. The values labelled "Exposed" show the binding of antibodies when each CTR was exposed or unmasked and therefore represent the amount of antibodies specific for each CTR. The signals "Masked" were the signals for antibody binding when the same positions were masked in the detection step. The difference in signal between "Exposed" and "Masked" represents the CTR specific antibodies. The signals show relative high variation on the CTRs compared to the complete serum (Figure 3.2a) and a peak is seen in for the CTRs Q90, N100, E110 and T114.

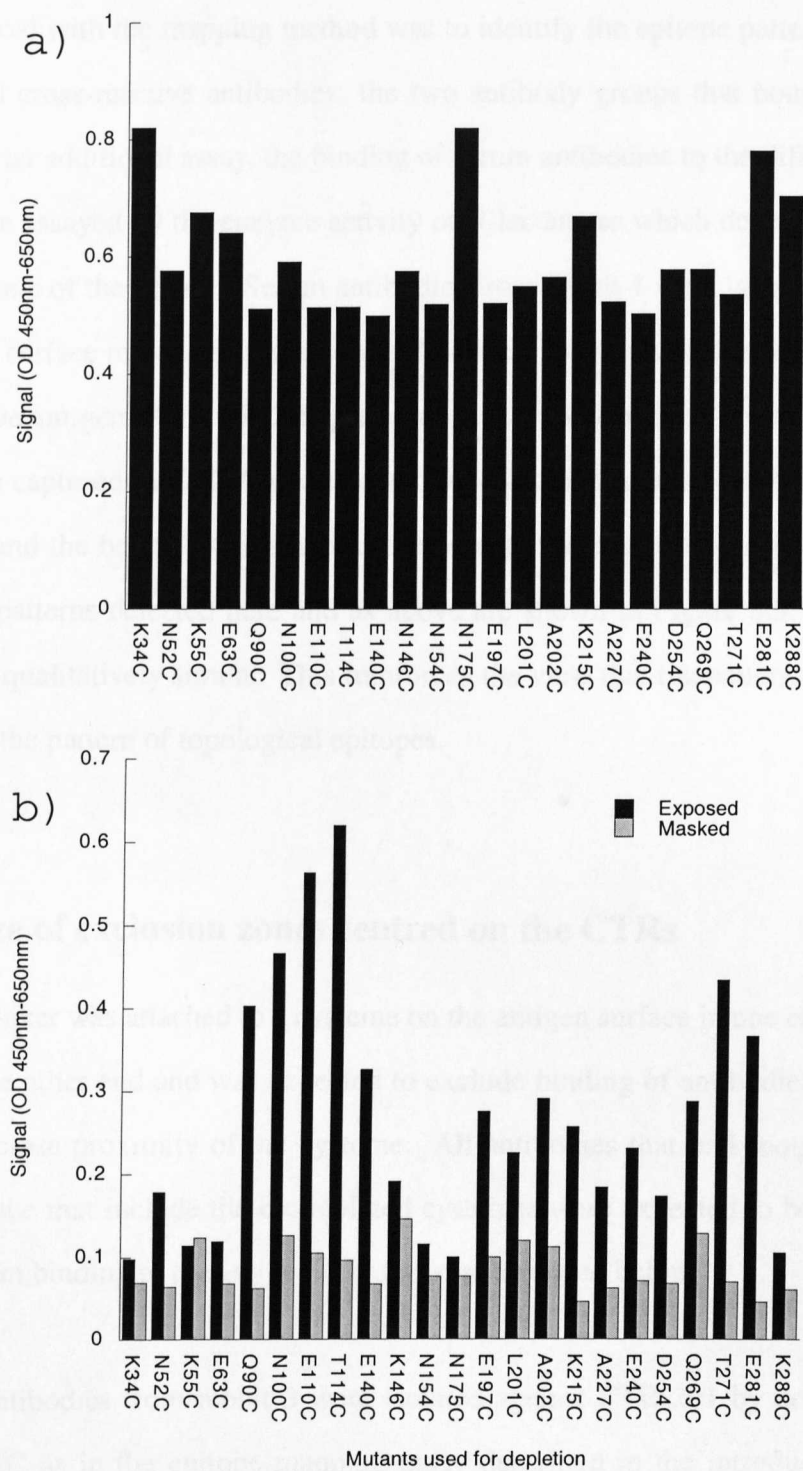


Figure 3.2: Image a) shows binding of serum antibodies (undepleted serum) to the biotinylated mutants. Image b) shows the mapped distribution of antibodies on the antigen surface for rabbit 1. The amount of antibodies detected to each region is displayed in black bars. The grey bars show the amount of antibodies to the remaining surface that were not successfully subtracted from the serum. The serum concentration in the mapping assay was 1:10,000.

### 3.2.2 Detection of region specific antibodies by enzyme activity

The main goal with the mapping method was to identify the epitope patterns of native-specific and cross-reactive antibodies; the two antibody groups that bound the native protein. As an additional assay, the binding of serum antibodies to the different surface regions were assayed by the enzyme activity of  $\beta$ -lactamase which depends entirely on the native state of the protein. Serum antibodies from rabbit 1 were isolated against the 23 different surface regions by depletion as described above and were then affinity purified on native antigen. After pH 2.5 elution and neutralisation the affinity purified antibodies were captured in ELISA wells coated with anti-rabbit antibodies.  $\beta$ -Lactamase was added and the bound  $\beta$ -lactamase detected with the chromogenic substrate Nitrocefin. The patterns detected here and as above are shown in Figure 3.3. The epitope patterns are qualitatively similar. This reinforces the view that the pattern detected corresponds to the pattern of topological epitopes.

### 3.2.3 Size of exclusion zones centred on the CTRs

The biotin-linker was attached to a cysteine on the antigen surface in one end and streptavidin in the other end and was expected to exclude binding of antibodies specific for epitopes in close proximity of the cysteine. All antibodies that had footprints on the antigen surface that include the biotinylated cysteines were expected to be completely excluded from binding to the antigen. This was investigated below.

Serum antibodies from rabbit 3 were isolated against CTR-201 by depletion with mutant L201C as in the epitope mapping assay described in the introduction of this chapter. These antibodies were then analysed for their ability to bind each of the 23 biotinylated  $\beta$ -lactamase mutants when captured on streptavidin coated ELISA plates. The results are shown in Figure 3.4. As expected, antibodies in the serum depleted with the biotinylated mutant L201C did not bind the same biotinylated mutant when captured on the ELISA plate. Low signals were also expected in the cysteine positions

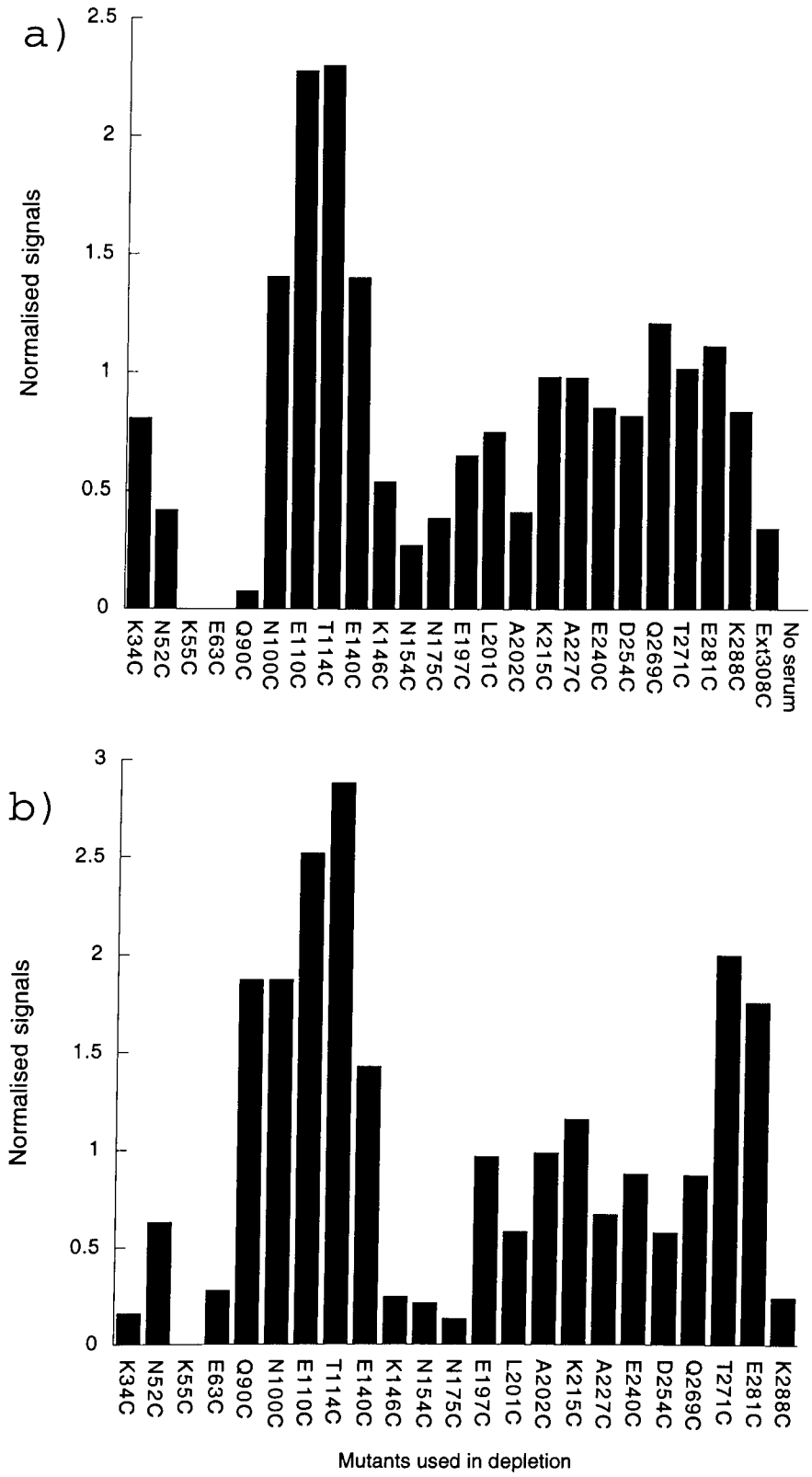


Figure 3.3: Serum antibodies from rabbit 1 which are detected by enzyme activity of  $\beta$ -lactamase (a) or with secondary antibodies (b). a) Serum antibodies specific to the 23 different regions of  $\beta$ -lactamase was isolated as described in the main mapping method. They were affinity purified on native protein before captured in ELISA plate wells coated with anti-rabbit antibodies.  $\beta$ -lactamase was then added in solution. The relative amount of bound native  $\beta$ -lactamase was then measured with a chromogenic  $\beta$ -lactamase substrate. The background (0.725) was relative high. b) The data in image are the same as in Figure 4.1a.

that were close to L201C, as the footprint of each antibody was expected to cover more than one of the cysteine positions on the surface. The strongest reduction was seen for A202C, E197C and N52C. These were also the closest positions (7.4, 10.4 and 15.8 Å from residue L201). The intermediate signals of other CTRs near L201C suggested a relation between the ability to block and the distance from the centre of the epitope. This is supported by the scatter plot in Figure 3.5. There was almost a linear relationship between signal and the distance from the surrounding CTRs to CTR-201 (the closest to the centre of the epitope). On the plot only distances less than 40 Å (measured by straight lines) are strictly meaningful due to the curvature of the protein. The data suggest a reduction to 40% of the maximum signal at 20 Å and a reduction to 20% of the maximum signal at 14 Å.

### **3.2.4 Depletion by antigen on plates: a way of reducing the signals on "Masked"**

Streptavidin coated beads were initially used for depletion in the mapping assay as they have a large surface area. They were therefore expected to efficiently bind and deplete serum antibodies. However, anti-serum from one of the rabbits (rabbit 2) contained antibodies that were not efficiently depleted with the antigen mutants on beads, but were detected on the same mutants captured on streptavidin coated ELISA plates as shown in Figure 3.6a. This suggests that the antiserum of rabbit 2 contained antibodies with low affinities that were difficult to deplete. However, when "High density" streptavidin coated ELISA plates were used for depletion of serum antibodies the depletion was successful (Figure 3.6b). This may reflect the great importance of the avidity of the antigen for capture of the low affinity serum antibodies.

The effect of immobilising the biotinylated  $\beta$ -lactamase mutants on beads versus plates in the depletion step were examined with one of the anti-sera that gave relatively low signals on "Masked" with both methods. The epitope patterns obtained with both methods are shown in Figure 3.7. The two methods gave similar data.

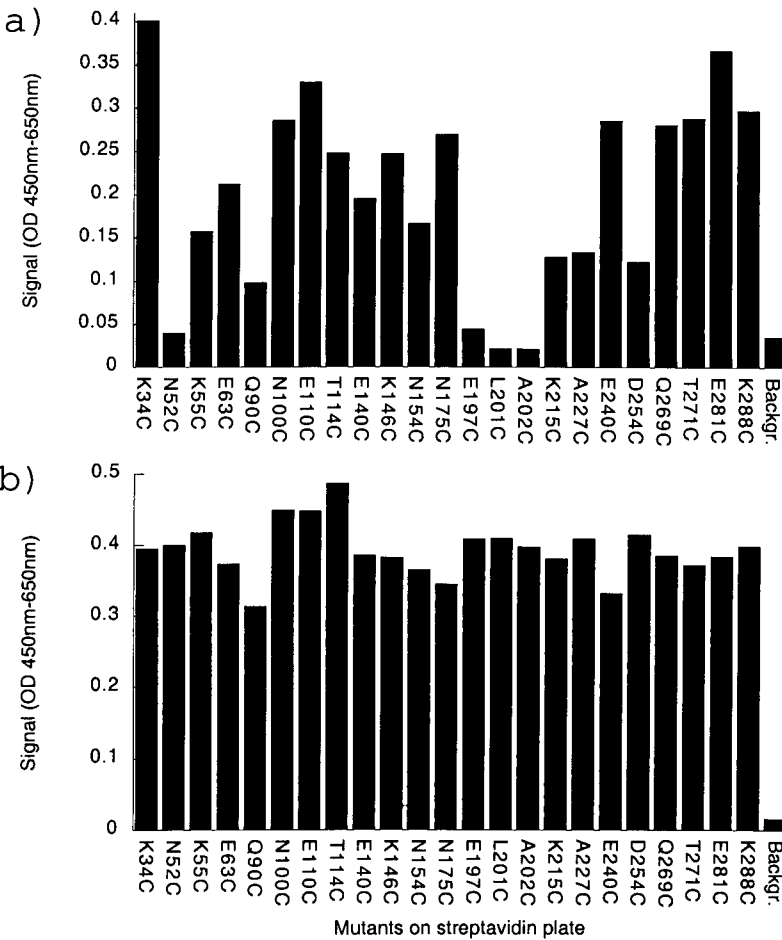


Figure 3.4: Resolution of mapping assay. a) Biotinylated  $\beta$ -lactamase mutants L201C on streptavidin beads were used to pull out (subtraction step) antibodies from the serum binding the  $\beta$ -lactamase surface that are not masked by the biotin-linker. The remaining antibodies were analysed for their ability to bind the panel of 23 biotinylated  $\beta$ -lactamase mutants when immobilised on streptavidin coated plates. b) Undepleted serum detected with the panel of 23  $\beta$ -lactamase mutants. The enzymatic detection was terminated after different time intervals to allow best internal comparison for the two data sets. The serum was from test-bleed three, rabbit 3. The dilution of the serum was 1:10,000 during the depletion step and 1:30,000 during detection of remaining antibodies.

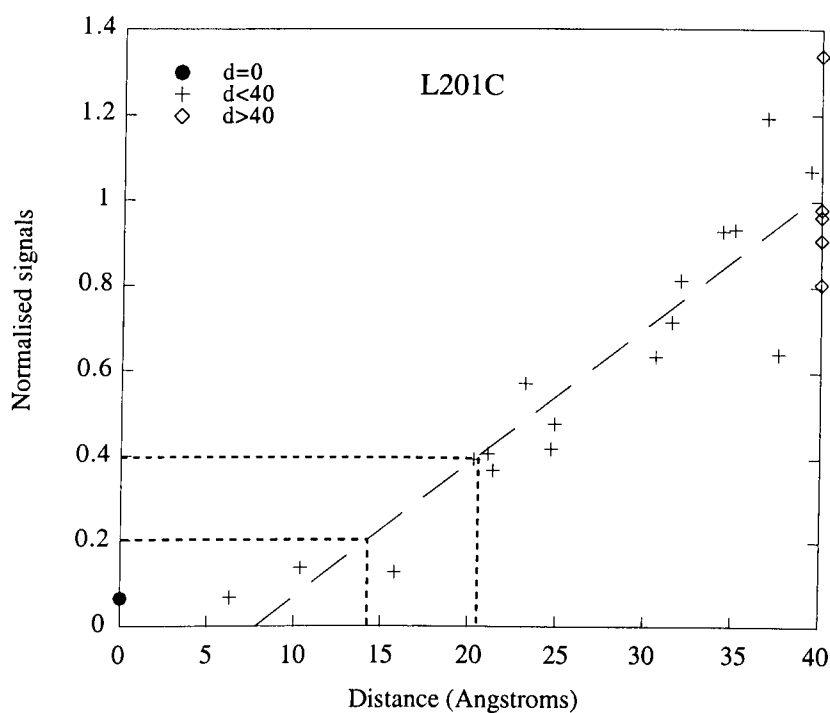


Figure 3.5: The resolution of the region specific depletion used in the mapping assay. The serum antibodies isolated against specific CTR-201 (Figure 3.4) positions as in the main mapping assay. Their ability to bind the different biotinylated  $\beta$ -lactamase mutants captured on a ELISA plate is plotted against the distance between the cysteine position used in the isolation process (depletion step) and in detection on the ELISA plate. The data are adjusted for the signal from whole serum (Figure 3.4b) and normalised to the average value for data points above 40 Å. The fitted line is given by the equation  $f = 0.032x - 0.25$  ( $R=0.93$ ).

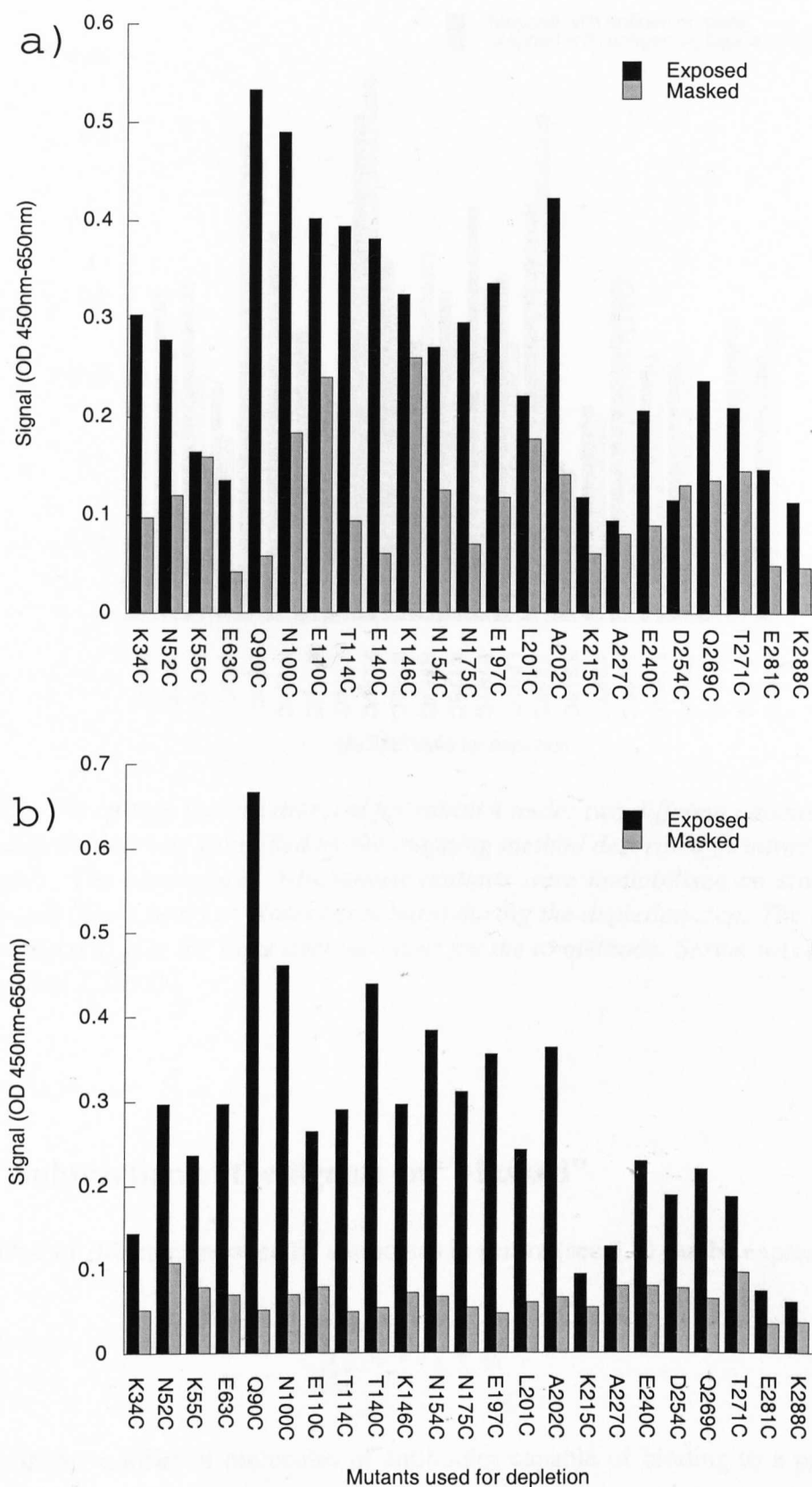


Figure 3.6: The epitope pattern detected for rabbit 2 under two different conditions. The epitope distribution was quantified by the mapping method described in introduction of this chapter. The biotinylated  $\beta$ -lactamase mutants were immobilised on streptavidin coated beads (a) or plates (b) during the depletion step. The amount of antibodies detected to each region is displayed in black bars (exposed). The grey bars (masked) show the amount of antibodies to the remaining surface that was not successfully subtracted from the serum.



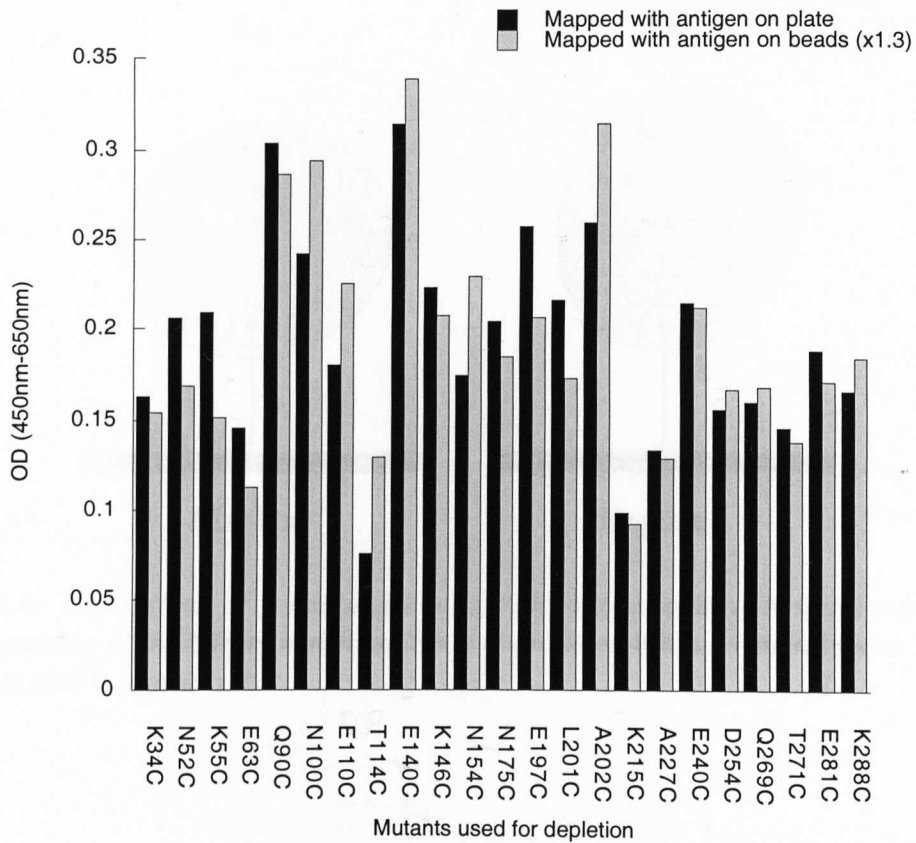


Figure 3.7: The epitope pattern detected for rabbit 4 under two different conditions. The epitope distribution was quantified by the mapping method described in introduction of this chapter. The biotinylated  $\beta$ -lactamase mutants were immobilised on streptavidin coated beads (black bars) or plates (grey bars) during the depletion step. The data sets are normalised to give the same average value for the two methods. Serum was used at a concentration 1:30,000.

### 3.2.5 Subtraction of the signals on "Masked"

The number of  $\beta$ -lactamase specific antibodies in serum (see 3.8) can be expressed as

$$\sum_{i=1}^{N-2} s_i + s_a + s_b \quad (3.1)$$

where  $s_i$  is the number of molecules of antibodies capable of binding to a patch  $i$  of the surface and  $s_a$  and  $s_b$  are the number of antibodies capable of binding patch  $a$  and  $b$  respectively. If the serum is then depleted with masked CTR-a then the number of antibodies capable of binding free antigen is

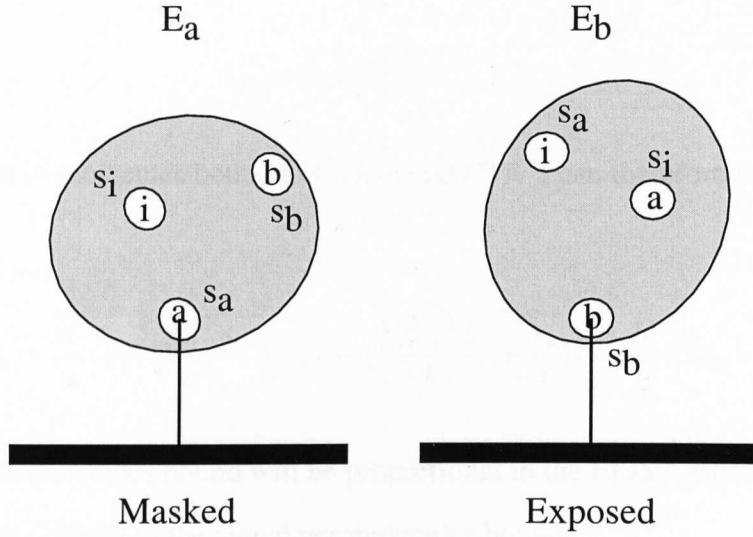


Figure 3.8: The surface of  $\beta$ -lactamase is divided into a total of  $N$  small patches including patches  $a$  and  $b$  from which antibody is excluded when covalently attached to a surface at  $a$  or  $b$  respectively.

$$\frac{\sum_{i=1}^{N-2} s_i}{k} + s_a + \frac{s_b}{k} \quad (3.2)$$

where  $k$  is the average depletion factor. However the number of antibodies available for capture when CTR-a is masked (and  $s_a = 0$ ) is

$$S_b = \frac{\sum_{i=1}^{N-2} s_i}{k} + \frac{s_b}{k} \quad (3.3)$$

and the number of antibodies available for capture when CTR-b is masked is

$$S_a = \frac{\sum_{i=1}^{N-2} s_i}{k} + s_a \quad (3.4)$$

With a number of molecules  $s$  and volume  $v$  the concentration  $c$  would be  $s/v$ . Assuming an equal binding constant  $K$  then the number of bound antibodies  $n = Kc = (Ks)/v$ . The number of molecules  $n_a$  and  $n_b$  bound on the antigen when CTR-a or CTR-b are masked respectively is then

$$n_a = \frac{K}{v} \cdot \left( \frac{\sum_{i=1}^{N-2} s_i}{k} + \frac{s_b}{k} \right) \quad (3.5)$$

and

$$n_b = \frac{K}{v} \cdot \left( \frac{\sum_{i=1}^{N-2} s_i}{k} + s_a \right) \quad (3.6)$$

The difference in molecules bound to CTR-b and CTR-a can therefore be described by

$$n_b - n_a = \frac{K}{v} \cdot \left( \left( \frac{\sum_{i=1}^{N-2} s_i}{k} + s_a \right) - \left( \frac{\sum_{i=1}^{N-2} s_i}{k} + \frac{s_b}{k} \right) \right) = \frac{K}{v} \cdot \left( s_a - \frac{s_b}{k} \right) \quad (3.7)$$

The number of molecules bound will be proportional to the ELISA signal  $E$ . This gives, with a constant  $C$  equal to the signal per molecules bound,

$$E_b - E_a = C(n_b - n_a) = C \left( \frac{K}{v} \cdot \left( s_a - \frac{s_b}{k} \right) \right) = C' \left( s_a - \frac{s_b}{k} \right) \quad (3.8)$$

Were  $C' = (CK)/v$ . The difference  $\Delta E_A = E_b - E_a$  describes the signal measured in the assay when a CTR-a is masked during depletion followed by detection on CTR-b (mutant 308C in the assay) minus the signal when detected on the same masked CDR (a).

Comparing the signals  $\Delta E_x$  and  $\Delta E_y$  for two adjacent patches  $x$  and  $y$  on the surface, the difference between these signals directly reflects the difference in number of antibody molecules binding to  $x$  and  $y$ . Thus

$$\Delta E_x - \Delta E_y = C' \left( s_x - \frac{s_b}{k} \right) - C' \left( s_y - \frac{s_b}{k} \right) = C' (s_x - s_y) \quad (3.9)$$

Note that this does not depend on the value of  $s_b$  or  $k$  as all the signals are taken with respect to the same reference point  $b$ . Furthermore, from equation 3.7 as

$$\Delta E_x = C' \left( s_x - \frac{s_b}{k} \right) \quad (3.10)$$

if  $s_x \gg \frac{s_b}{k}$ , then  $\Delta E_x = C' s_x$ , likewise  $\Delta E_y = C' s_y$

$$\Rightarrow \frac{\Delta E_x}{\Delta E_y} = \frac{C' s_x}{C' s_y} = \frac{s_x}{s_y} \quad (3.11)$$

In this case the ratios of the signals  $\Delta E_x$  to  $\Delta E_y$  would reflect directly the ratios of

antibodies that bind each patch  $x$  and  $y$ .

The experiment achieves  $s_x \gg \frac{s_b}{k}$ . An upper limit for the value of  $s_b$  ( $s_{308C}$ ) was determined for rabbit 1 by depleting the serum with CTR-308 followed by detection on CTR-90, and vice versa. The signal to the former was about 1/6 of the latter (data not shown), and therefore the value  $s_b$  is low (and lower than the average). Furthermore the depletion factor  $k$  was large; thus the reduction of the signals on "masked" CTRs in the mapping assay was at least a 12 times smaller with depleted serum compared to undepleted serum for rabbit 1 (data not shown). Hence  $s_x \gg \frac{s_b}{k}$ .

In subsequent chapters (chapter 4, 5 and 6) signals for masked CTR has been subtracted from that of unmasked CTR.

### 3.2.6 Reproducibility of the epitope mapping assay

The epitope mapping assay had a high degree of reproducibility. This is illustrated in Figure 3.9. The epitope distribution in the same serum was determined in two separate experiments. 15 of biotinylated mutants were used in the mapping assay. The serum concentrations were two-fold different in the two experiments. The signals from these two sets samples were developed enzymatically in two separate assays and were therefore displayed with the same average value (by multiplying the signals with grey bars by 1.12). The epitope patterns are almost identical. Similar reproducibility was also observed when the antigen mutants were displayed on beads during the depletion.

## 3.3 Discussion

The epitope assay provided identical conditions for detecting antibodies specific for any part of the antigen surface. Antibodies specific for each of the 23 CTRs were detected on the wild type antigen and the data collected allowed comparison of the antibody response to the different parts of the antigen surface. Previous assays for mapping epitopes on na-

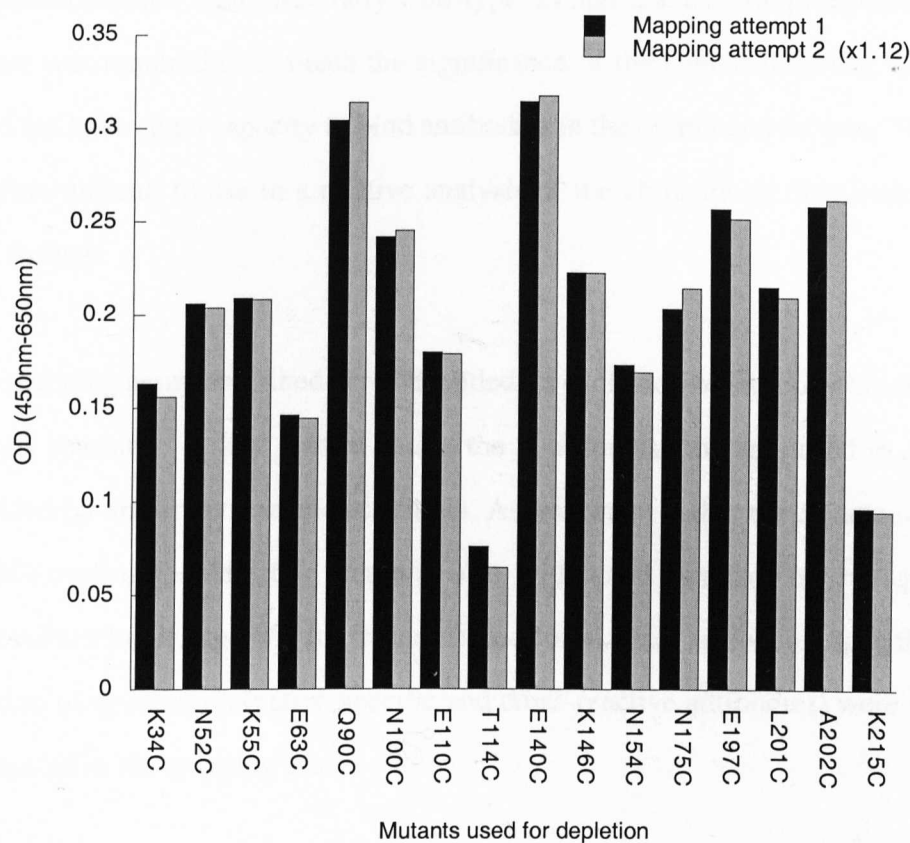


Figure 3.9: The mapping of surface distribution of antibodies to 15 different surface regions. The two columns for each region are done in separate experiments. The serum dilutions were 1:12,500 and 1:25,000 for the black and the grey bars respectively. The two sets of signals are displayed with the same average value (by multiplying signals with grey bars with 1.12). Serum from rabbit 4.

tive proteins using sera have depended on exclusion of antibody binding to the antigen surface by conservative variations in the surface residues. However this would not necessarily exclude antibodies, or indeed exclude antibodies equally well in different surface regions. The readout and interpretation of such assays is very complex, as seen with Homologous Scanning Mutagenesis used by Weber and his colleagues [Weber et al., 1992] (see chapter 1). For example the antigen specific antibodies were detected when bound to the mutants (rather than essentially wild-type, as here), and a complex normalisation procedure was required to evaluate the significance of the signals detected, as the mutants did not have equal capacity to bind antibodies in the unmutated regions. This assay is therefore difficult to use in a relative analysis of the antigenicity distribution on the antigen surface.

The mapping assay described here depended on exclusion of antibody binding to the antigen by masking. A key feature is that the  $\beta$ -lactamase mutants used in depletion were folded (as shown by enzyme activities). As important is that the  $\beta$ -lactamase (mutant 308C) used in the detection step was also folded and therefore not recognised by monoclonal antibodies specific for the denatured form. Only antibodies specific for the native form of the antigen (native-specific and cross-reactive antibodies) were expected to be detected in the mapping assay.

The results from the mapping assay using the enzyme activity of  $\beta$ -lactamase readout also supports the supposition that the epitope distribution was that on native protein. Only native  $\beta$ -lactamase that bound the serum antibodies could contribute to the signal as denatured  $\beta$ -lactamase had no enzyme activity. Even though the signal was low in this assay and the background relatively high, the general epitope distribution detected were qualitatively the same as detected in the main mapping assay. The format of the assay may have had some influence on the detected pattern. The binding was not enhanced by avidity effect and the relative contributions to the signals by the antibodies with high affinity were therefore expected to be higher. Some of the antibodies may also have inhibited the enzyme activity of  $\beta$ -lactamase and therefore not contributed to the signal.

A mix of 1:100 of this rabbit serum (rabbit 1) in PBS with  $0.1\mu\text{g/ml}$   $\beta$ -lactamase gave a 35% reduction of the enzyme activity compared to a  $\beta$ -lactamase solution without serum present (data not shown). The introduced cysteines with the shortest surface distance to the active site were N100C, E110C, K215C, E240C, Q269C and T271C. Antibodies against these regions could potentially have inhibited the enzyme activity.

The epitope mapping assay does not identify specific surface residues on the antigen that were directly involved in antibody binding but topological regions. The size of the regions was investigated by the following experiment (see Figures 3.4 and 3.5) in which each of the CTRs was used for detection rather than a single CTR (CTR-308). The serum was first depleted with one cysteine mutant (CTR-201) and then assayed for binding to the panel of 23 other CTRs. The readout depends on two exclusion processes. The first (during the depletion) leaves in solution antibodies excluded by CTR-201. The second exclusion is during the detection step. The results show (Figure 3.5) that the signal for serum antibodies to CTR-201 is significantly reduced only if the distance between the centre of CTR-201 and the other CTRs is  $< 15 \text{ \AA}$ , which is about the radius of the footprint of an antibody ( $(\pi^{-1} \cdot 850 \text{ \AA}^2)^{\frac{1}{2}} \approx 16 \text{ \AA}$ ). This means that only antibodies with footprints that were very close to or overlapping with the cysteine position had been excluded in CTR-201 and were present after the depletion step.

The relative epitope distribution could in principle be determined by using undepleted serum to the panel of biotinylated and immobilised  $\beta$ -lactamase mutants. The differences in signals would show the contribution of antibodies to the different CTRs (see equation 3.1). In practise however, these differences are submerged in experimental noise. Efficient depletion of the antibodies was therefore necessary to get accurate estimates of epitope distribution.

The mapping assay was based on depletion by the masked antigen. However, low affinity antibodies are not expected to be depleted. Indeed the sera from rabbit 2 gave high signals binding on the masked antigen in the detection step, suggesting incomplete

capture. To deal with this, avidity effects were harnessed for efficient depletion. Streptavidin coated beads rather than plates were initially used for depletion as it was surmised that the beads would provide a larger surface area. The streptavidin beads with biotinylated mutants provided a surface area of  $24 \text{ cm}^2$ . However multiple streptavidin coated ELISA plate wells with in which the sera could subsequently be depleted on immobilised biotinylated antigen provided a similar surface area ( $11 \text{ cm}^2 \approx 11 \times 0.95 \text{ cm}^2$ ). Using "High density" streptavidin coated ELISA plates provided good conditions for capture of the antibodies as supported by the results.

The conditions for binding the antibodies to the antigen during the detection step did not necessarily need to match the conditions during the depletion step. The key requirement was that if the affinity of an antibody was too low to be depleted, then the detection assay should be designed to be insensitive to binding of such a low affinity antibody.

The epitope patterns represent combinations of concentrations and affinities of the serum antibodies. The concentrations of the antibodies were probably a major contribution to the epitope pattern as discussed later. The distribution of epitope affinities on the antigen surface or the epitope distribution of only high affinity antibodies would give important additional information about the epitope distribution. Surface plasmon resonance (Biacore) was therefore used in an attempt to measure affinities of the serum antibodies. However the serum concentrations were too low to get detectable signals with the Biacore even at high densities of antigen on the chip.



# Chapter 4

## Mapping epitopes of serum antibodies

### 4.1 Introduction

The epitope mapping method presented in chapter 3 generates data on the relative epitope distribution on the native protein. The method was used in this chapter to compare the epitope distributions of sera from different animals.

### 4.2 Results

#### 4.2.1 Immunisation and epitope mapping

Two rabbits (rabbits 1 and 2) were immunised with  $\beta$ -lactamase in Freund's adjuvant (see Table 4.1). Both animals were injected with the antigen in Freund's complete adjuvant (FCA) followed by boosts with the antigen in Freund's incomplete adjuvant (FIA). The epitope distribution in the sera were analysed subtracting the signals "Masked" from the signals "Exposed" as discussed in chapter 3. The serum from rabbit 2 was mapped with the biotinylated  $\beta$ -lactamase immobilised on plates in stead of beads to reduce the high signals on "Masked" as described earlier. The results are shown in Figure 4.1. (Raw data are presented in Figure A.2, page 148.) There were relatively large variations in the amount of antibodies over the surface. There were regions with almost three times the average signal as seen for CTR-114 in rabbit 1 and CTR-90 in rabbit 2. There were also six to seven CTRs with less than half the average signal. The CTRs with high or low

Table 4.1: *Immunisation regimes. All the animals were each immunisation day injected subcutaneously at four sites on the back. The rabbits were injected with  $\beta$ -lactamase in 500  $\mu$ l PBS alone or mixed with 500  $\mu$ l Freund's Complete Adjuvant (FCA) or Freund's Incomplete Adjuvant (FIA). The barstar was in 100  $\mu$ l PBS mixed with 100  $\mu$ l FCA or FIA. The mice were injected with  $\beta$ -lactamase in 100  $\mu$ l PBS mixed with 100  $\mu$ l FCA or FIA. The test-bleeds were taken 10 or 7 days after the antigen injections for the rabbits or the mice respectively. Rabbit 5 was injected with carboxymethylated (CM)  $\beta$ -lactamase. Rabbits 3, 4, and 5 are described in later chapters.*

Animal	Antigen	1st injection Day 1	1st boost Day 29	2nd boost Day 57
Rabbit 1	$\beta$ -lactamase	500 $\mu$ g in FCA	500 $\mu$ g in FIA	500 $\mu$ g in FIA
Rabbit 2	$\beta$ -lactamase	500 $\mu$ g in FCA	500 $\mu$ g in FIA	500 $\mu$ g in FIA
Rabbit 3	$\beta$ -lactamase	500 $\mu$ g in PBS	500 $\mu$ g in PBS	500 $\mu$ g in PBS
Rabbit 4	$\beta$ -lactamase	500 $\mu$ g in PBS	500 $\mu$ g in PBS	500 $\mu$ g in PBS
	+ barstar	100 $\mu$ g in FCA	100 $\mu$ g in FIA	100 $\mu$ g in FIA
Rabbit 5	CM- $\beta$ -lactamase	500 $\mu$ g in FCA	500 $\mu$ g in FIA	500 $\mu$ g in FIA

		Day 1	Day 22	Day 43
Mouse 1	$\beta$ -lactamase	250 $\mu$ g in FCA	30 $\mu$ g in FIA	20 $\mu$ g in FIA
Mouse 2	$\beta$ -lactamase	250 $\mu$ g in FCA	30 $\mu$ g in FIA	20 $\mu$ g in FIA
Mouse 3	$\beta$ -lactamase	250 $\mu$ g in FCA	30 $\mu$ g in FIA	20 $\mu$ g in FIA
Mouse 4	$\beta$ -lactamase	250 $\mu$ g in FCA	30 $\mu$ g in FIA	20 $\mu$ g in FIA

signals seemed to group together in the linear sequence for rabbit 1. The two clusters with high signals were from Q90 to E140 and T271 to E281. The intervals with low signals were from K34 to E63C and N146 to N175. The results from rabbit 2 also indicated some clusters of low signals close to the C-terminus. The highest signal in this rabbit (on Q90) fell within the cluster with highest signals in rabbit 1.

The same epitope data were plotted on the protein surface to get a better picture of the three-dimensional distribution as shown in Figure 4.2. The CTRs with high signals for rabbit 1 form two clusters on the surface of the protein (red, orange and yellow). There was also a large continuous surface region with low signals (blue and violet). The CTR with highest signal for rabbit 2 (CTR-90) was only 23 Å away from the CTR with highest signal in rabbit 1 (CTR-114) on the protein surface. The regions with low and intermediate signals were not in the same surface regions for the two rabbits.

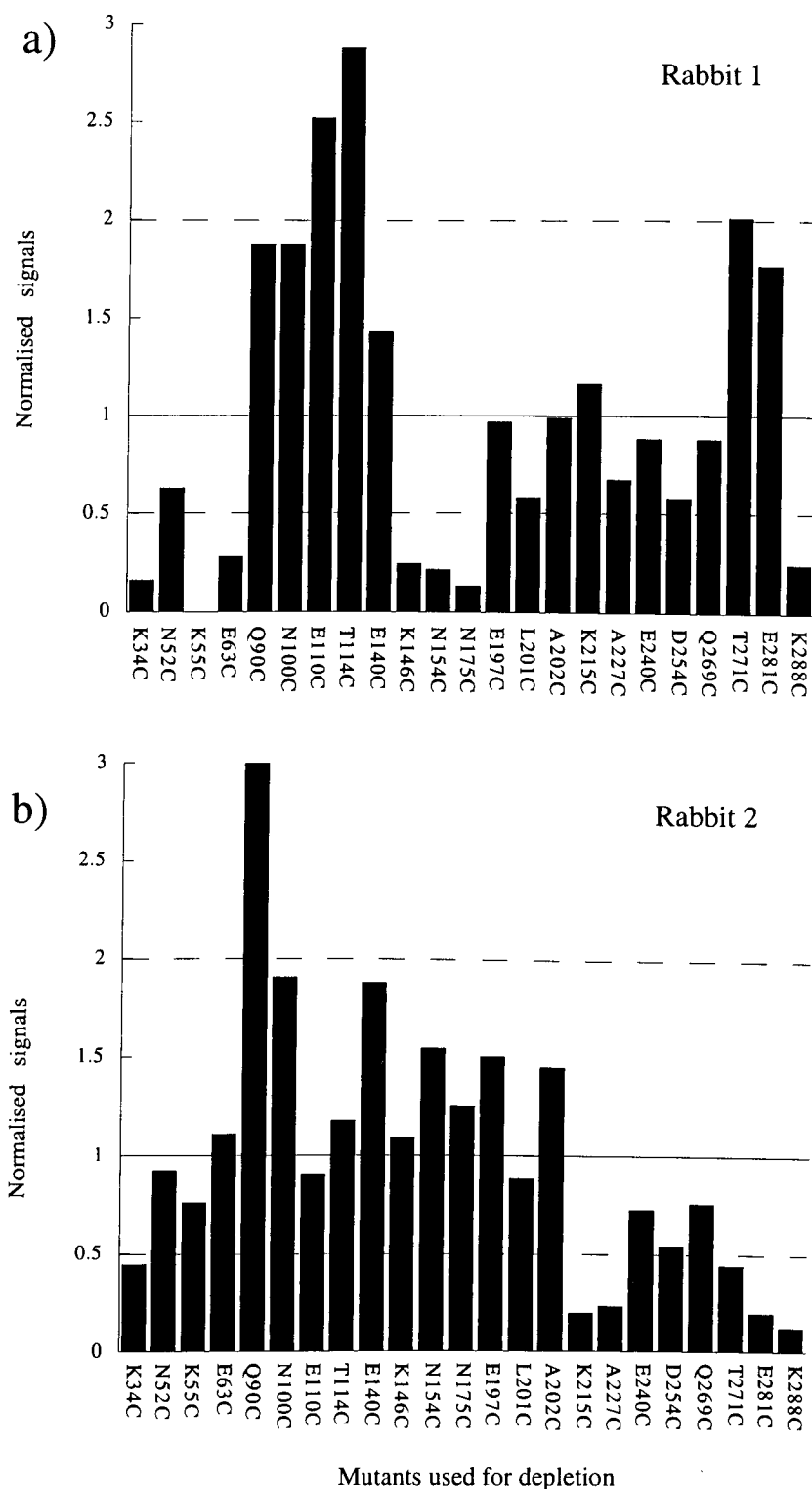


Figure 4.1: The relative distribution of serum antibodies to different surface locations. The results for rabbit 1 and 2 are shown in image (a) and (b) respectively. The signals are normalised to give an average of 1. The signals on "Masked" are subtracted and negative values are set to zero. The average value is indicated with a full line. The dotted lines indicate half the average signal and double the average signal. The sera concentrations in the mapping assay were 1:10,000 and 1:50,000 for rabbit 1 and 2. The serum from rabbit 2 was mapped with the biotinylated  $\beta$ -lactamase immobilised on plates in stead of beads to reduce the high signals on "Masked" as described in chapter 3. The rabbits primarily injected with antigen in FCA followed by antigen in FIA in first and second boost.

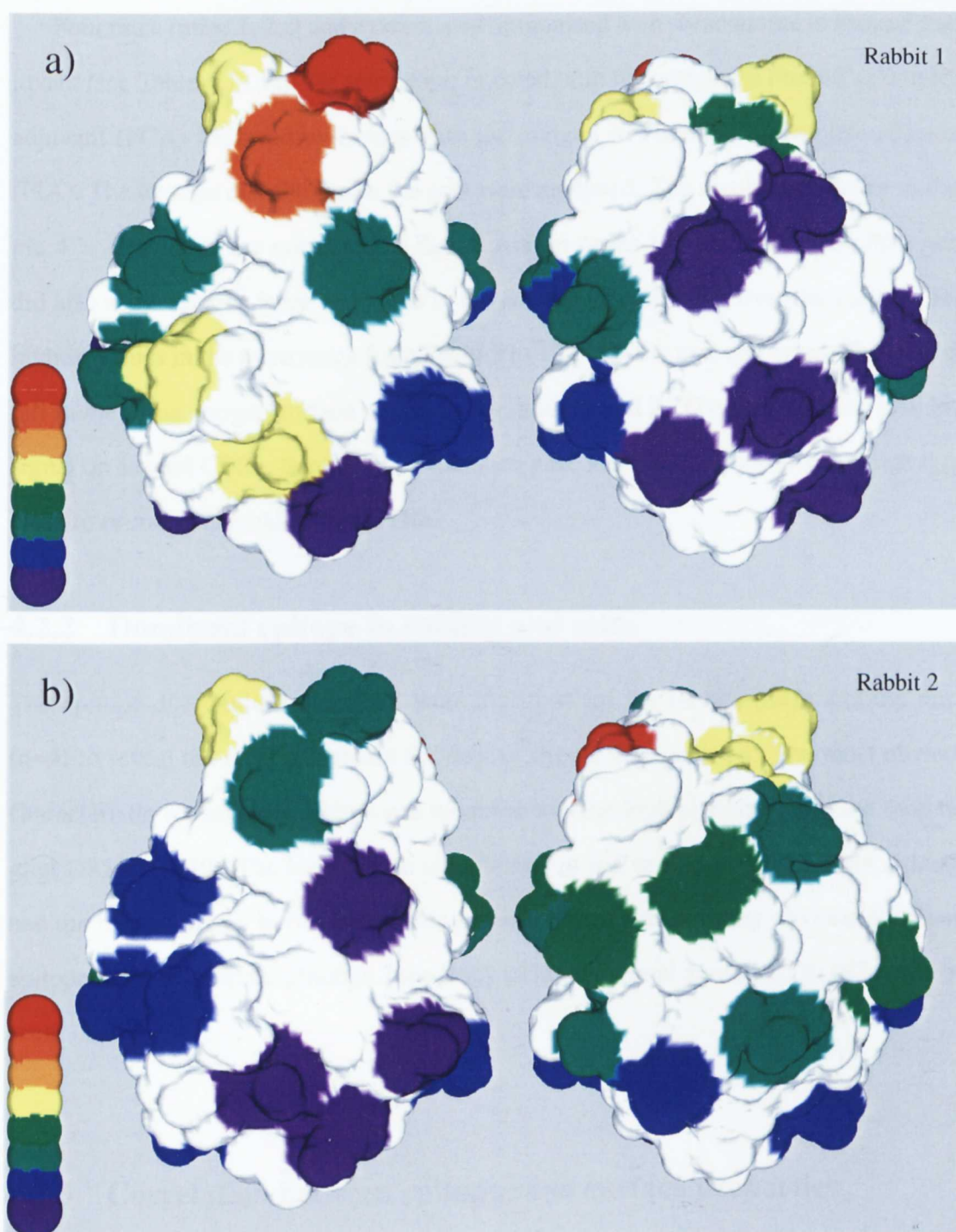


Figure 4.2: The epitope distributions of sera antibodies from rabbits 1 and 2 on the three-dimensional surface of  $\beta$ -lactamase are shown in image (a) and (b) respectively. The protein is rotated  $180^\circ$  around the vertical axis in the two images. The background colour of the protein is white. The surface around each cysteine position (sulphydryl group), in a radius of seven Ångströms, is coloured according to the relative amount of antibodies detected to the particular region. The values for the detected antibodies are translated into the colours of the rainbow with regular intervals. Red corresponds to the highest value and violet corresponds to the lowest value. The signals on "Masked" are subtracted. Note that the regions coloured around each detection point are significantly smaller than the typical footprint of an antibody.

Four mice (mice 1, 2, 3 and 4) were also immunised with  $\beta$ -lactamase in Freund's adjuvant (see Table 4.1). All the mice were injected with the antigen in Freund's complete adjuvant (FCA) followed by boosts with the antigen in Freund's incomplete adjuvant (FIA). The epitope distribution in the sera were analysed. The results are shown in Figure 4.3. (Raw data are presented in Figure A.3 on pages 149 and 150.) The mice sera did also show relative large variations in the amount of antibodies over the surface. The highest values in the mice range from about 5 to 12 times the average value. They did all fall in the same antigen surface region as for rabbit 1 and 2. The sera did also give low signal on several CTRs. Sera from all the mice gave less than half the average signal on close to or more than half of the CTRs.

#### 4.2.2 Dominant epitope in rabbits and mice

The epitope distributions described were averaged for the rabbits ( $n=2$ ) and the mice ( $n=4$ ) to reveal the most prominent features as shown in Figure 4.4. The most obvious characteristic for both the rabbits and the mice was the high signals within the loop region D85 to V119. The loop region is indicated in red in Figure 4.6. All six animals had the highest signal in this part of the protein. There was no other obvious dominant epitope in these animals although a tendency of higher signal around CTR-197 could be seen.

#### 4.2.3 Correlation between epitopes and surface properties

The rabbits immunised with  $\beta$ -lactamase in Freund's adjuvant all had highest antibody response against the loop region D85 to V119 shown in Figure 4.6. Loops tend to have high flexibility. Indeed the CTRs in the interval D85 to V119 have the highest backbone mobility as seen in Figure 4.7. The average polypeptide backbone flexibility (crystallographic B-values, [Jelsch et al., 1993]) corresponding to a window of seven residues around each CTR were checked for correlation with the ELISA signals. This gave correlation coefficients of 0.60 and 0.61 for the average rabbit and mouse signals or 0.70 and

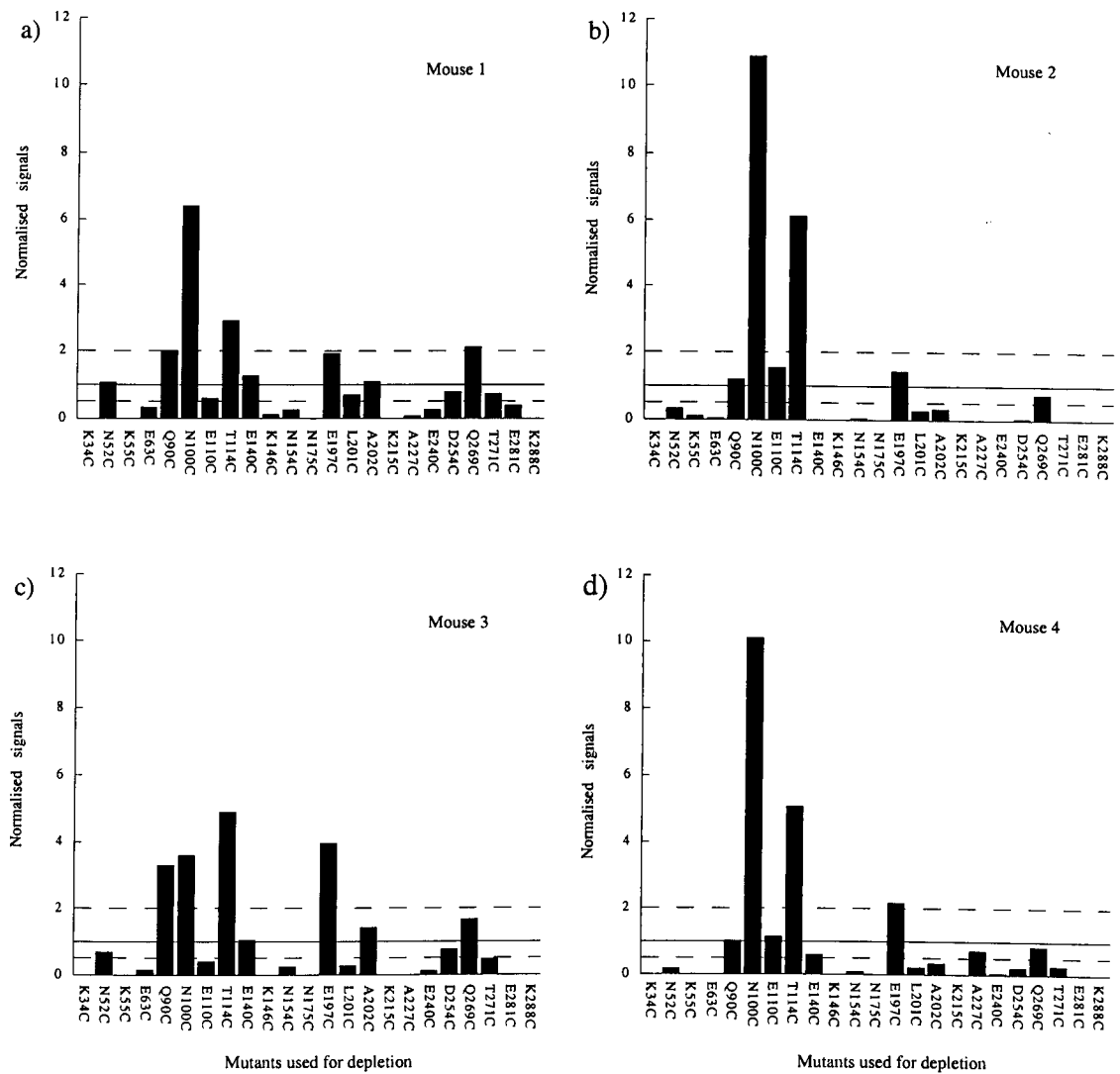


Figure 4.3: The relative distribution of serum antibodies to different surface locations for sera from mice. The signals are normalised to give an average of 1. The signals on "Masked" are subtracted. The average is indicated with a full line. The dotted lines indicate half the average signal and double the average signal. The serum concentration in the mapping assay was 1:5,000. The mice (mouse 1, 2, 3 and four in image (a), (b), (c) and (d) respectively) were primarily injected with antigen in FCA followed by antigen in FIA in first and second boost.

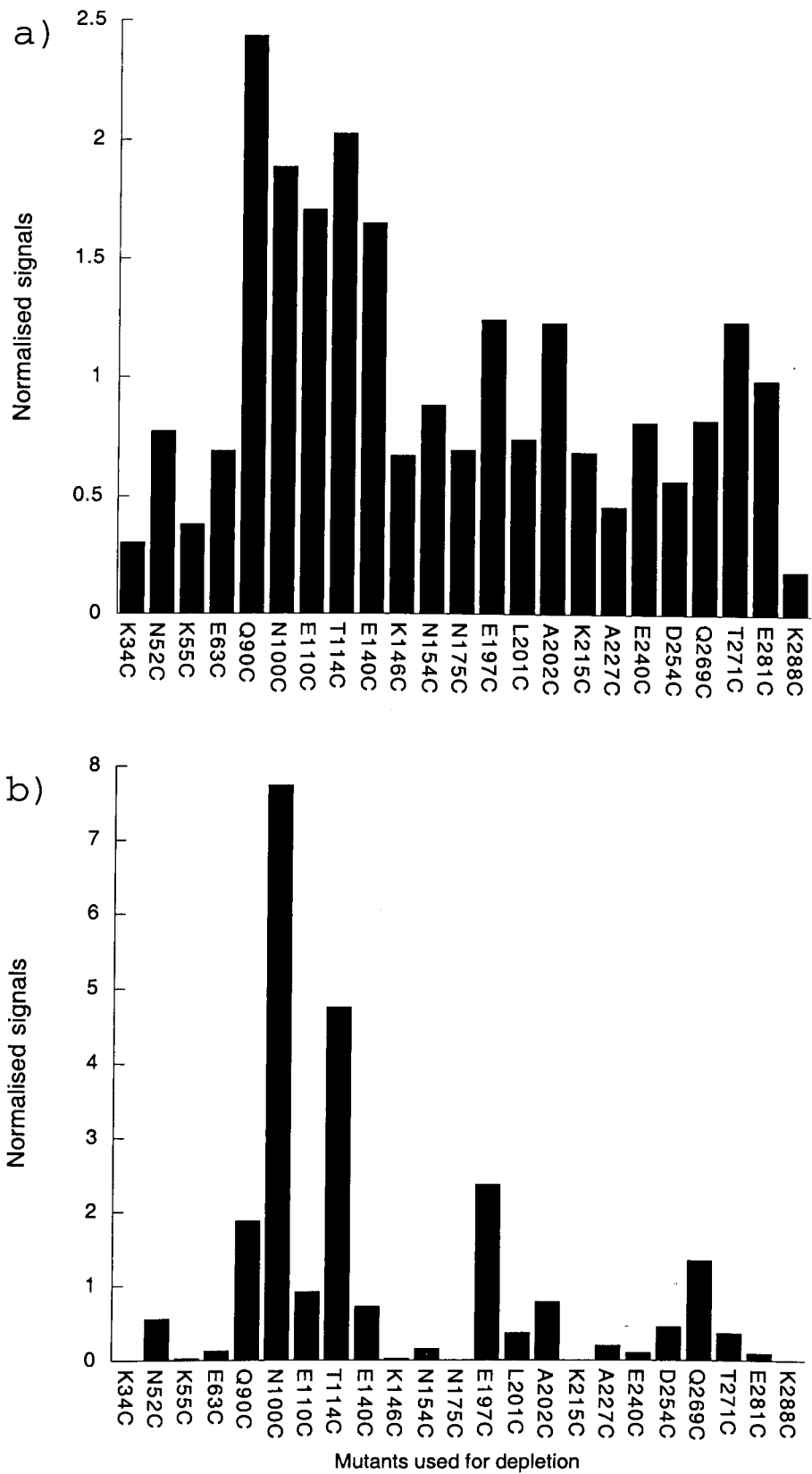


Figure 4.4: The average epitope distributions of for rabbits 1 and 2 in image (a) and mice 1, 2, 3 and 4 in image (b). The signals are normalise to an average value of 1.0.

0.46 if the epitope data from the same animals were weighted by using the exponential ELISA signals. (Correlation data for all the animals are shown in Table A.1 and A.2, pages 152 and 153.) This suggests that there is a positive correlation between ELISA signals and the backbone mobility. Scatter plots with the backbone flexibility and ELISA signals are shown in Figure 4.5.

Figure 4.8 shows how the sequences with highest backbone mobility locate on the surface. The red surface in this figure includes the CTRs with highest signals for the rabbits immunised with Freund's adjuvant. The epitope data are not based on the linear sequence but on the three-dimensional surface. A refinement of the correlation calculations was therefore carried out by calculating the average flexibility (crystallographic B-values) of the polypeptide backbone attached to side chains comprising a CTR. For this purpose each CTR was assumed to include all surface residues within a 10 Å radius centred on each sulphhydryl of the cysteine mutants. This gave correlation coefficients of 0.71 and 0.61 for the average rabbit and mouse signals or 0.74 and 0.47 if the epitope data from the same animals were weighted by using the exponential ELISA signals.

### 4.3 Discussion

The results described here are the first examples in the literature of relative epitope data on a three-dimensional protein surface. The systematic data allows a more a thorough analysis and comparison of the antibody responses than earlier epitope data.

The results are consistent with earlier results for other protein antigens. Most if not all the antigen surface was antigenic as also described earlier by Benjamin et al. [1984]. The sera from all the animals immunised with  $\beta$ -lactamase further showed highest antibody response in the loop region D85 to V119. The epitope dominance seen for the loop region D85 to V119 in all the sera is unlikely to be the result of a random antibody response against the different surface regions. A large part of the earlier literature reports



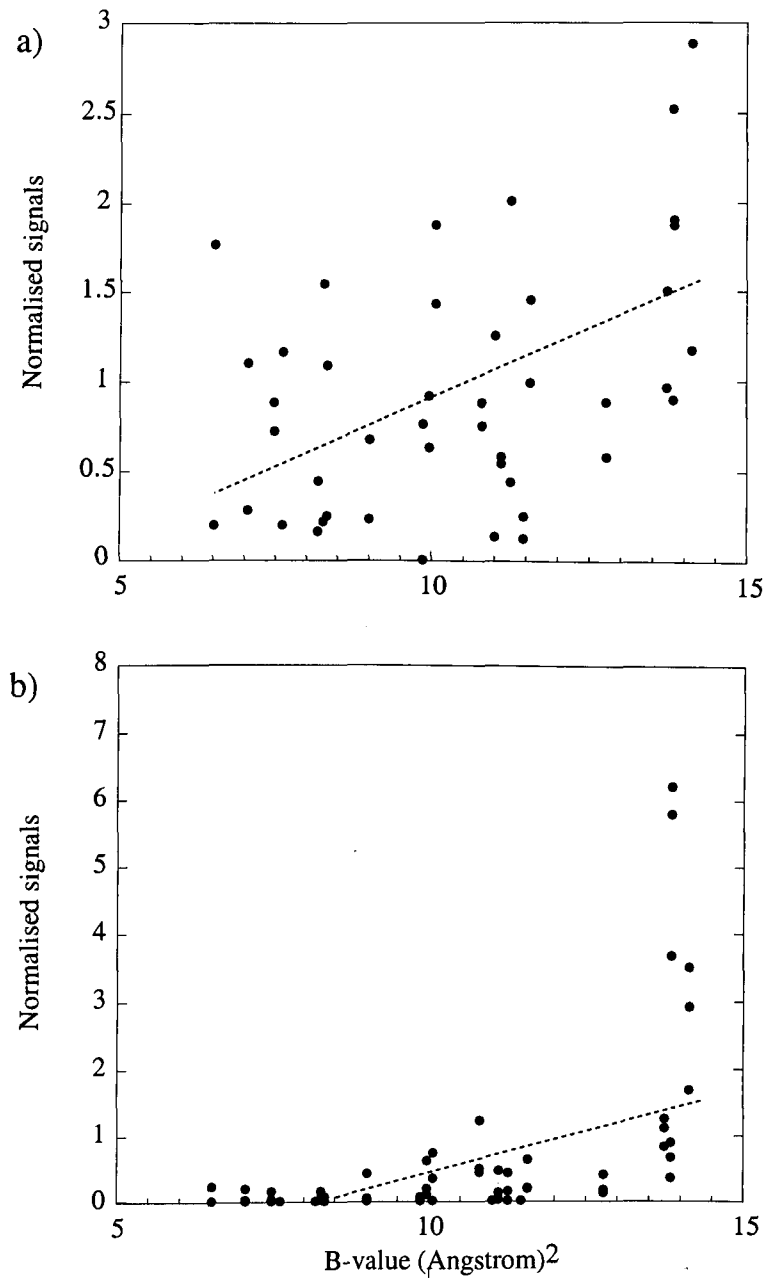


Figure 4.5: Scatter plot of the *ELISA* signals and average backbone mobility for a window of seven amino acids around the CTRs for the rabbits ( $n=2$ ) in image a and the mice ( $n=4$ ) in image b.

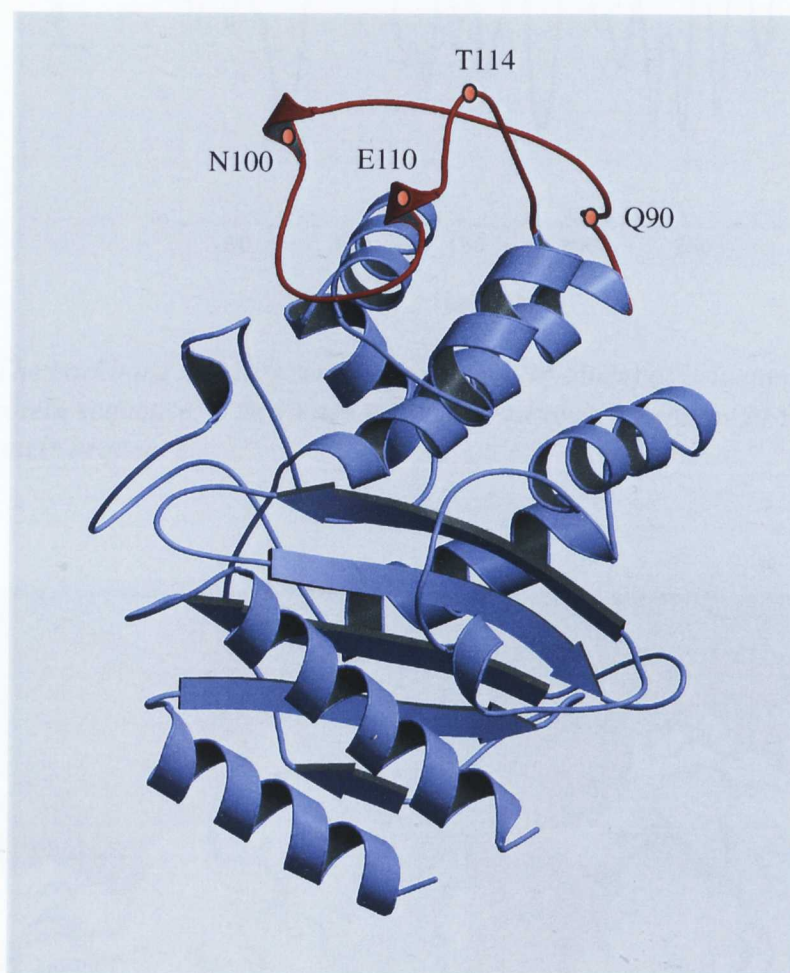


Figure 4.6:  $\beta$ -Lactamase is shown in light blue, except the flexible loop region D85 to V119 which is in red. The protein is shown from the same angle as in the left images of the Figures 4.2 and 5.3. Sera from all the animals immunised with  $\beta$ -lactamase in Freund's adjuvant showed highest antibody response in this region of the protein.

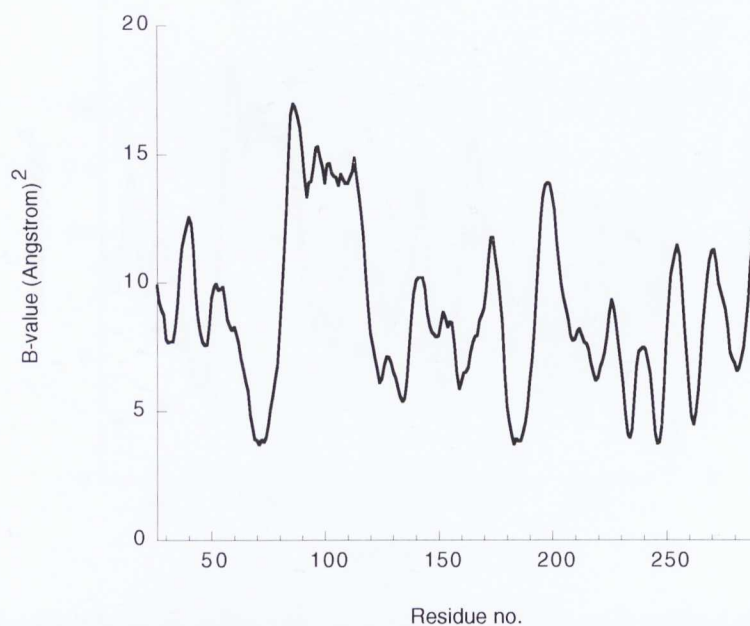


Figure 4.7: The backbone mobility (crystallographic B-values) of  $\beta$ -lactamase is plotted along the protein sequence. The values are averaged over a window of seven residues along the protein sequence.

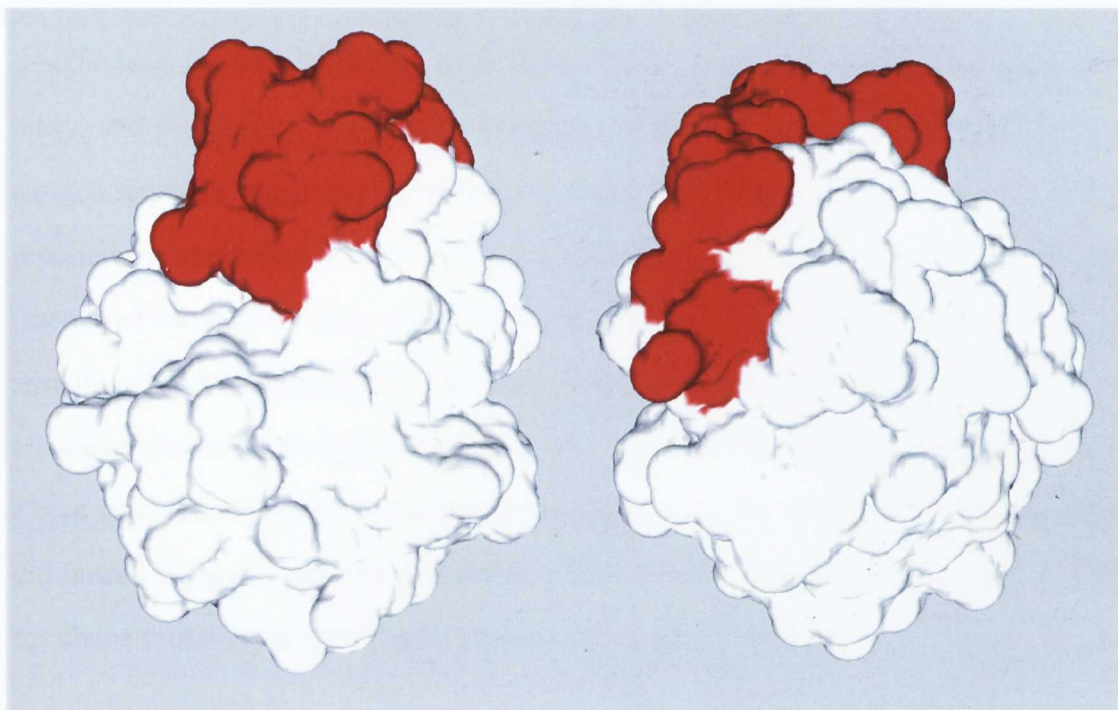


Figure 4.8: The  $\beta$ -lactamase residues with the highest backbone mobility (B-values above  $12 \text{ \AA}^2$ , see Figure 4.7) are coloured in red on a white background. The protein is shown from the same angles as in the presentations of the mapping data earlier in this chapter.

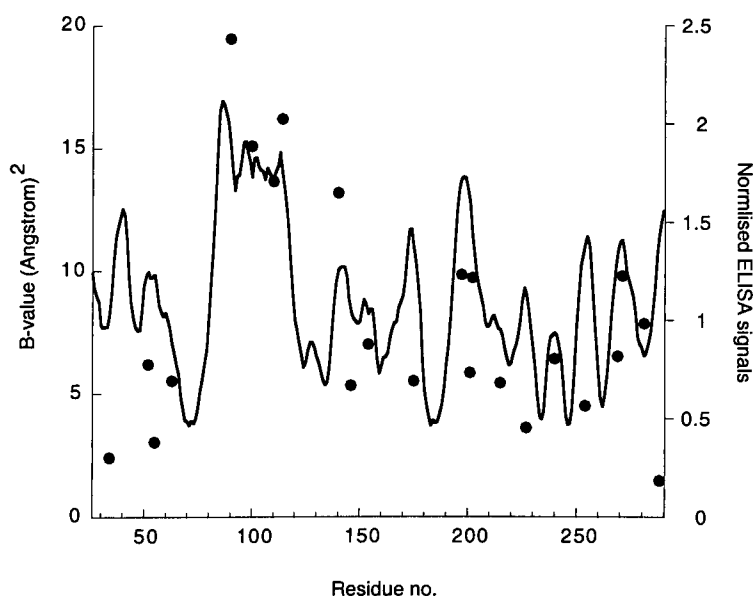


Figure 4.9: *The backbone mobility (B-values) of  $\beta$ -lactamase is plotted along the protein sequence together with the normalised values for rabbits 1 and 2 (average for the two rabbits). The B-values are averaged over a window of seven residues along the protein sequence.*

similar epitope dominance as mentioned in chapter 1.

The loop region D85 to V119 has little secondary structure and high backbone mobility, and the profiles of epitope distribution and backbone mobility along the linear antigen sequence suggested that they were connected (Figure 4.9). Westhof et al. [1984] reported the same kind of relation between the backbone mobility and the antigenicity (measured using small linear peptides) for the model antigens tobacco mosaic virus protein, myoglobin and lysozyme. The relative values from the mapping assay reported here allowed a numerical analysis in contrast to the visual inspection used in the earlier work. These calculations based on the backbone mobility in the sequence around the CTRs did indeed suggest a positive correlation. This was also supported by the analysing the backbone mobility for topographic regions of the antigen surface.

## 4.4 Conclusion

The antibodies produced in response to  $\beta$ -lactamase in Freund's adjuvant had a relatively uneven distribution on the antigen surface. All the animals immunised with  $\beta$ -lactamase produced antibodies predominantly to the surface region with the highest backbone flexibility, the loop region D85 to V119.

## Chapter 5

# Epitope distribution without Freund's adjuvant

### 5.1 Introduction

The results in chapter 4 describe the presence of a dominant epitope on  $\beta$ -lactamase that correlate with a region of high flexibility. The results were based on sera from animals which were all immunised with antigen in Freund's adjuvant. This raises questions whether the presence of Freund's adjuvant could influence the epitope distribution and if so, whether it was associated with the dominant epitope. These are important questions since almost all earlier epitope studies with model antigens have been carried out with this adjuvant. Here immunisation was undertaken with antigen in only phosphate buffered saline (PBS).

#### 5.1.1 Immunisation with protein in PBS

Two rabbits (rabbits 3 and 4) were immunised with  $\beta$ -lactamase without Freund's adjuvant (see Table 4.1). The animals were injected three times subcutaneously with  $\beta$ -lactamase in PBS. Rabbit 4 was in addition given the bacterial protein barstar in Freund's adjuvant in a separate injection with the intention to evoke an antibody response to the native  $\beta$ -lactamase. The lipopolysaccharide (LPS) content in the  $\beta$ -lactamase sample was measured to see if there were other chemicals in the protein sample with adjuvant

activity [Johnson et al., 1956]. The analysis was carried out by BioWhittaker, Europe. The samples contained 21.34 EU per millilitre, equivalent to 2.13ng LPS per millilitre. Hence each injection given to the rabbits contained about 1ng LPS. (This also included the rabbits immunised with  $\beta$ -lactamase in Freund's adjuvant.) It is difficult to know if the LPS at nanogram levels as used in the current work had a significant adjuvant effect as no comparative study had been undertaken in the literature. Both the rabbits immunised with  $\beta$ -lactamase in PBS produced antibodies (IgG) specific for  $\beta$ -lactamase. The ELISA results with test-bleeds are shown in Figure A.1, page 146. The titres of the responses in these animals were comparable to the response with Freund's adjuvant. It is not known if the additional injection of barstar in Freund's adjuvant in rabbit 4 had any adjuvant effect as both rabbits 3 and 4 made antibodies to the native  $\beta$ -lactamase. The sera of the two rabbits immunised with  $\beta$ -lactamase in PBS had increased titers of IgG which is typical for secondary immune responses [Bandilla et al., 1969]. Two mice were also immunised with  $\beta$ -lactamase in PBS. However the mice did not produce  $\beta$ -lactamase specific antibodies (IgG) in detectable concentrations.

The epitope distributions in the sera from the rabbits were examined by the mapping method. The results are shown in the Figure 5.1. (Raw data are presented in Figure A.4, page 151.) There are few extreme values in these histograms compared to Figure 4.1 with results from the rabbits immunised with the same antigen in Freund's adjuvant. The serum from rabbit 3 gave less than half the average signal on only two CTRs and there were no CTRs with more than twice the average signal. The serum from rabbit 4 gave less than half the average signal on only one CTR and there was no CTR with more than twice the average signal.

The epitope distributions for the two rabbits immunised with  $\beta$ -lactamase in PBS are averaged in Figure 5.2. There is no obvious dominating epitope in this figure. The signals were in general closer to the average signal than for the sera from the animals immunised with Freund's adjuvant. The epitope in loop region D85 to V119 observed in all six animals immunised with  $\beta$ -lactamase in Freund's adjuvant did not dominate the

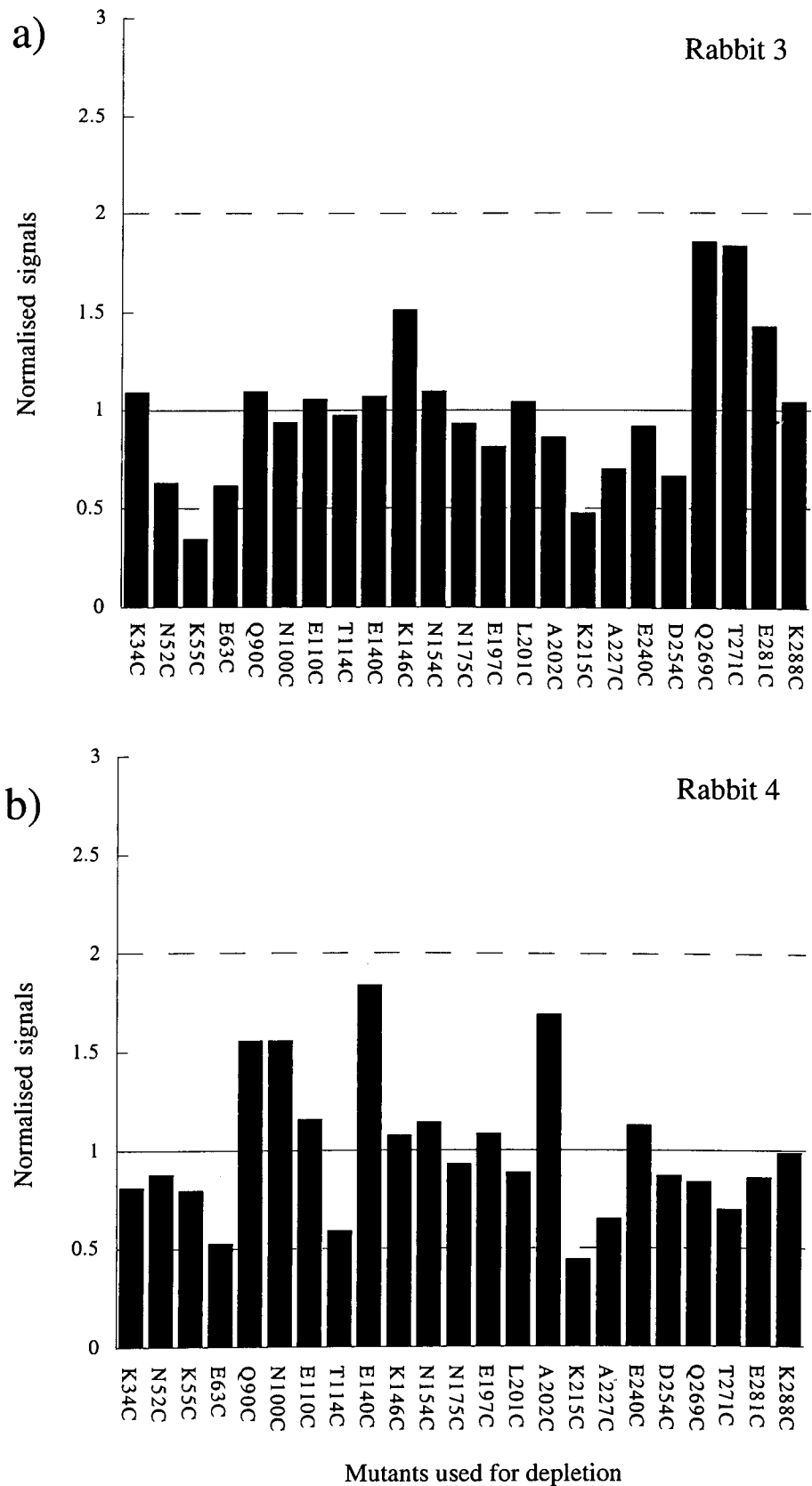


Figure 5.1: The relative distribution of serum antibodies to different surface locations. The results for rabbit 3 and 4 are shown in image (a) and (b) respectively. The signals are normalised to give an average of 1. The signals on "Masked" are subtracted. The average is indicated with a full line. The dotted lines indicate half the average signal and double the average signal. The serum concentration in the mapping assay was 1:30,000 for both rabbits. The rabbits were given three injections with antigen in PBS.



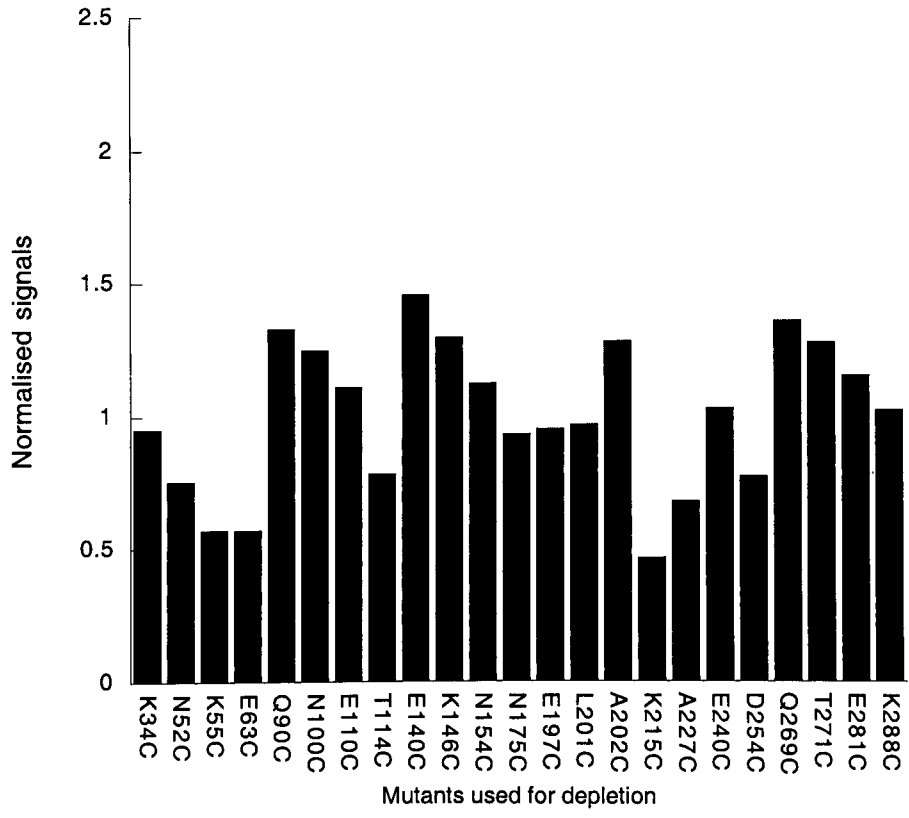


Figure 5.2: The average epitope distributions of for the rabbits 3 and 4, both immunised with  $\beta$ -lactamase in PBS. The signals are normalise to an average value of 1.0.

antibody response in any of the rabbits immunised with  $\beta$ -lactamase in PBS.

The surface epitope data for rabbit 3 and 4 are presented on the three-dimensional antigen surface in Figure 5.3. The signals were, as expected from the histograms, relatively even on the surface. The region with the highest signals for rabbit 3 (CTR-269 and CTR-271) did not fall in the same surface region as for the rabbits immunised with antigen in Freund's adjuvant. The CTR with highest signal for rabbit 4 (CTR-140) was spatially close to the CTRs with highest signals for all the animals immunised with  $\beta$ -lactamase in Freund's adjuvant. But the signal in rabbit 4 was relatively low compared to the more extreme values for the sera from the animals immunised with  $\beta$ -lactamase in Freund's adjuvant.

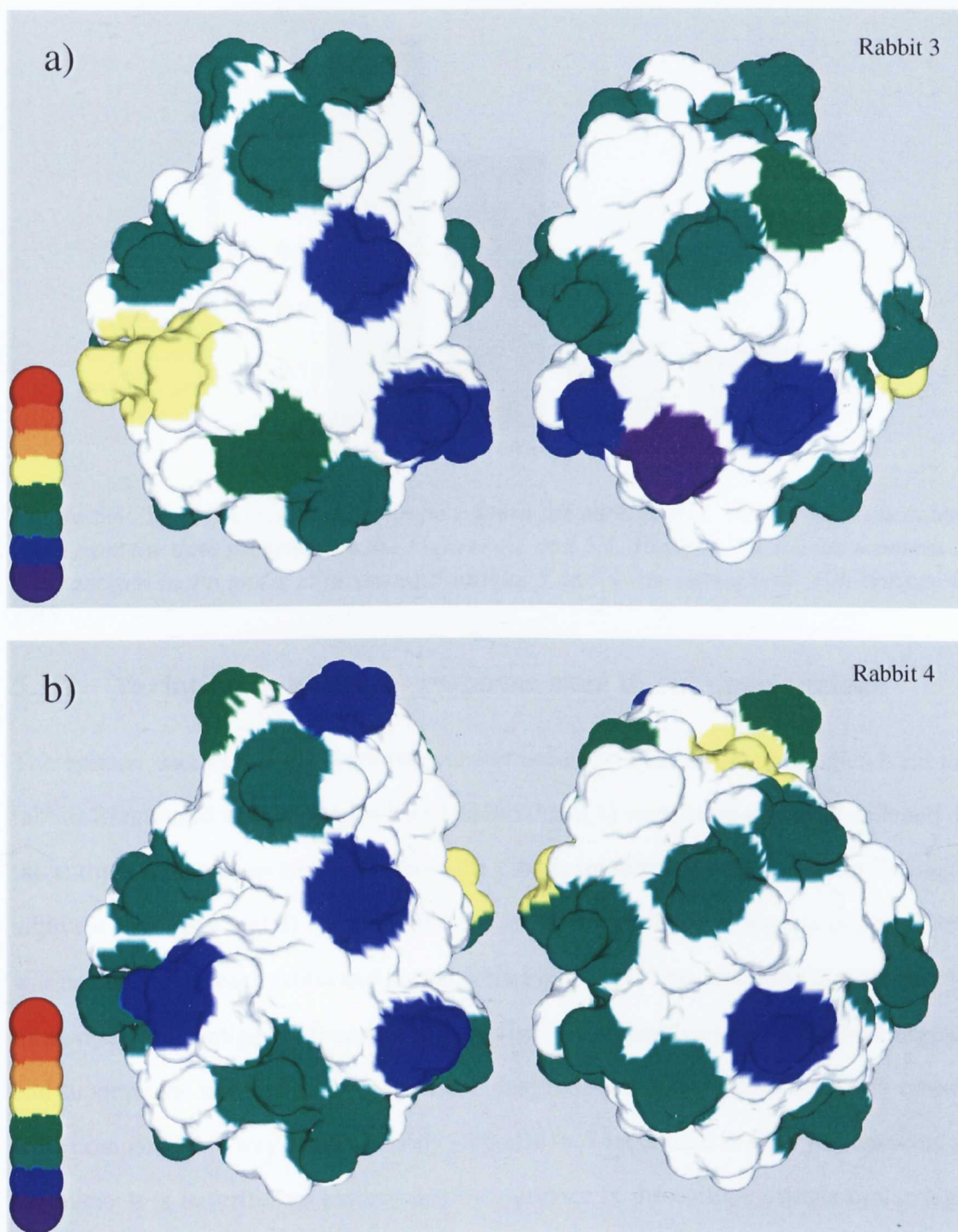


Figure 5.3: The epitope distributions of sera antibodies from rabbits 3 and 4 on the three-dimensional surface of  $\beta$ -lactamase are shown in image (a) and (b) respectively. The protein is rotated 180° around the vertical axis in the two images. The background colour of the protein is white. The surface around each cysteine position (sulphydryl group), in a radius of seven Ångströms, is coloured according to the relative amount of antibodies detected to the particular region. The values for the detected antibodies are translated into the colours of the rainbow with regular intervals. Red corresponds to the highest value and violet corresponds to the lowest value. The signals on "Masked" are subtracted. Note that the regions coloured around each detection point are significantly smaller than the typical footprint of an antibody.

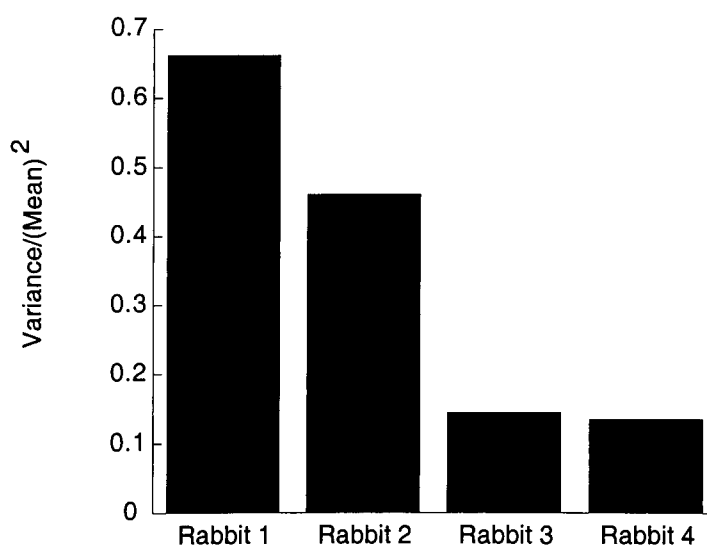


Figure 5.4: The variance in the epitope pattern for each rabbit. The variance is calculated from the data presented in the Figures 4.1 and 5.1. Rabbits 1 and 2 are immunised with antigen in Freund's adjuvant and rabbits 3 and 4 are immunised with antigen in PBS.

### 5.1.2 Variation in antibody response over the antigen surface

The epitope data in this and previous chapter indicated that serum antibodies from the rabbits immunised with antigen in PBS (rabbits 3 and 4) were more evenly distributed on the antigen surface than antibodies from the rabbits immunised with antigen in Freund's adjuvant (rabbits 1 and 2). A numerical value for the variance in signals on the CDRs was calculated for each rabbit and presented in Figure 5.4. The data show that the rabbits immunised with antigen in Freund's adjuvant had the highest variation in epitope signals and supports the impression from the visual inspection of epitope patterns. Only rabbits were compared this way as no antibody response to  $\beta$ -lactamase in PBS was detected in the mice. It is nevertheless evident that the variance in the epitope distribution is high for the four mice immunised with  $\beta$ -lactamase in Freund's adjuvant.

### 5.1.3 Correlation between epitopes and surface properties

The average polypeptide backbone flexibility (crystallographic B-values) corresponding to a window of seven residues around each CTR were checked for correlation with the average ELISA signals for rabbit 3 and 4 in the same way as described in chapter 4. This

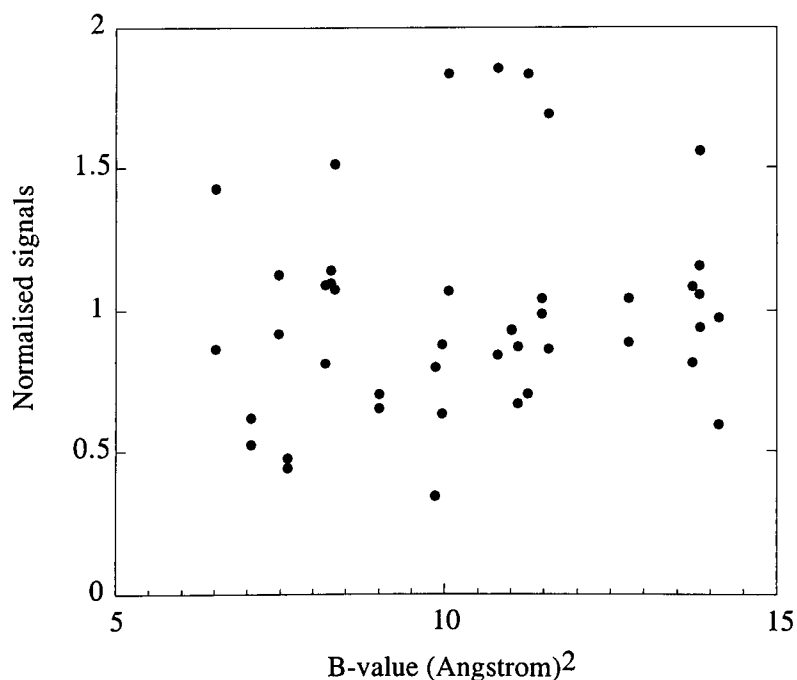


Figure 5.5: Scatter plot of the ELISA signals and average backbone mobility for a window of seven amino acids around the CTRs for the rabbits 3 and 4. Both were immunised with  $\beta$ -lactamase in PBS and are combined in the figure.

gave a correlation coefficient of 0.28 or 0.24 if the epitope data from the same animals were weighted by using the exponential ELISA signals. There is also no obvious trend in the scatter plot of the antigenicity and backbone flexibility for the rabbits immunised with  $\beta$ -lactamase in PBS (Figure 5.5) as suspected with the presented correlation data.

The correlation coefficients for the average backbone flexibility in the surface regions around the CTRs (see chapter 4) and the ELISA signals were 0.33 or 0.03 if the epitope data from the same animals were weighted by using the exponential ELISA signals. In contrast, the weighted ELISA signals for the rabbits immunised with  $\beta$ -lactamase in Freund's adjuvant (rabbits 1 and 2) showed correlation coefficient of 0.74 with the backbone flexibility.

### 5.1.4 Signals on "Masked" in mapping assay

The ratio of "Exposed"/"Masked" was systematically higher for the rabbits immunised with  $\beta$ -lactamase in PBS compared to the rabbits immunised with  $\beta$ -lactamase in Freund's adjuvant (Figures A.2 and A.4, pages 148 and 151). The ratios were about 10 and 11 for the rabbits immunised with  $\beta$ -lactamase in PBS compared to 3 and 4 for the rabbits immunised with  $\beta$ -lactamase in Freund's adjuvant. The lower ratios (or higher signals on "Masked") in the latter rabbits are expected to be due to low affinity antibodies as they were not successfully depleted from the serum in the depletion step but were detected on the ELISA plates (see chapter 3). The low affinity antibodies may have been a result of antibody responses to unfolded protein as discussed in more detail in the next chapter.

## 5.2 Discussion

The rabbits immunised with  $\beta$ -lactamase in PBS did not reveal any dominant epitope. There was also no indication of elevated signal in the loop region D85 to V119 for the sera from these rabbits, the loop region that was dominant in all the animals immunised with  $\beta$ -lactamase in Freund's adjuvant. These results suggest that the epitope distribution was dependent on the immunisation regime used although sera from only a few animals were analysed. The antibodies from the rabbits immunised with the antigen in PBS were further relatively evenly spread on the antigen surface. Each of the sera from the rabbits immunised with  $\beta$ -lactamase in Freund's adjuvant had relative high variation in antibody response to the different parts of the antigen surface. This difference in epitope variations with different immunisation regimes also suggests a dependence of the antigenicity distribution on the immunisation method.

The epitope distributions in sera from the rabbits immunised with  $\beta$ -lactamase in PBS showed little if any correlation with backbone mobility, in contrast to the sera from the rabbits immunised with  $\beta$ -lactamase in Freund's adjuvant. This suggests that cor-

relation between dominant epitopes and backbone mobility reported earlier for tobacco mosaic virus protein, myoglobin and lysozyme [Westhof et al., 1984] may also have been artefacts of using Freund's adjuvant which they used in the immunisation.

### 5.3 Conclusion

Immunisation with  $\beta$ -lactamase in PBS induced production of antibodies with relatively even distributions on the whole antigen surface. The dominant epitope seen in sera from the animals immunised with  $\beta$ -lactamase in Freund's was absent in the rabbits immunised with  $\beta$ -lactamase in PBS. Only a weak correlation was detected between the epitope distributions in the rabbits immunised with  $\beta$ -lactamase in PBS and the backbone flexibility.

# Chapter 6

## State of the antigen in Freund's adjuvant

### 6.1 Introduction

The adjuvant we today know as Freund's complete adjuvant is a mineral oil with a surfactant and heat killed mycobacterium. Freund's incomplete adjuvant is the same solution without the mycobacterium. The oil-based adjuvant is mixed with the antigen in a water-based buffer to create a stable water-in-oil emulsion which is injected into the animals. Jules Freund showed in 1937 [Freund, 1937, 1947] that administering the antigen in these emulsions stimulate stronger antibody responses than injecting the antigen in a water based buffer.

A few papers describe Freund's adjuvant's impact on the state of protein antigens although with different conclusions. Scibienski [1973] immunised guinea pigs with hen egg lysozyme (HEL) with or without adjuvant and found that antibodies induced without adjuvant bound native but not denatured protein while antibodies induced in the presence of Freund's adjuvant bound both forms of the protein. This suggests that Freund's adjuvant denatured HEL. Berzofsky et al. [1976] studied human hemoglobin (Hb) in Freund's adjuvant by electromagnetic resonance. The resonance spectrum of the heme group is dependent on the conformation of the protein. The data showed no denaturation

of Hb and the different results for HEL and Hb indicate that the denaturation effect of the adjuvant may depend on the specific protein.

Although some have argued that possible denaturation of protein antigens by Freund's adjuvant may have implications for the distribution of epitopes (Stanworth's comment in Van Regenmortel et al. [1986]), most researchers studying epitopes have shown little concern about the state of the antigens in the adjuvant. The results in the chapters 4 and 5 of this thesis show that the epitope distributions on  $\beta$ -lactamase were different if this antigen was injected with Freund's adjuvant or only in PBS. The impact of Freund's adjuvant on the state of  $\beta$ -lactamase and how this may influence the epitope distribution was therefore investigated in this chapter. These issues are of great importance since Freund's adjuvant has been used in the vast majority of earlier epitope studies with model antigens.

## 6.2 Results

### 6.2.1 Different ways of emulsifying the protein in Freund's adjuvant

A classic way of mixing Freund's adjuvant with the antigen is to manually push the solution between two interconnected glass syringes [Weir, 1978]. As manually mixing of the adjuvant is not necessarily reproducible, emulsification by a magnetic spinner and a homogeniser, both powered by electrical engines, were evaluated. The emulsions created by these two methods did not pass the classic test described in Weir [1978], the emulsion should stay as compact droplets when added to water, and the emulsions were therefore mixed by the classic method described above.

### 6.2.2 Assaying denaturation by enzyme activity of $\beta$ -lactamase

The enzyme activity of  $\beta$ -lactamase is dependent on the native conformation of the polypeptide chain. The most obvious way to evaluate the state of  $\beta$ -lactamase in Fre-



und's adjuvant was therefore to add a chromogenic substrate (Nitrocefin) to the adjuvant emulsion and measure the enzyme activity. But the Nitrocefin was efficiently absorbed into the oil phase and not the water phase where native  $\beta$ -lactamase was expected to be. The water phase was therefore separated from the adjuvant followed by analysis of the included  $\beta$ -lactamase. Ether, hexane or mineral oil was used to destabilise the emulsions which could then be separated by centrifugation. Freund's incomplete adjuvant was used in these experiments.

### **Incubation time and enzyme activity**

The water phase rescued from the emulsion had only small amounts of enzyme activity compared to the enzyme activity of the  $\beta$ -lactamase put into the emulsion. Using ether, hexane or mineral oil made no obvious difference. But some variations were observed in the remaining enzyme activity which seemed to depend on the incubation time in the adjuvant. Ether, which destabilised the emulsion more efficiently than hexane or oil, was used to analyse the time dependence as it soon became apparent that short incubation times in the adjuvant were needed to rescue reasonable amounts of native  $\beta$ -lactamase. The enzyme activities in the water phase after different incubation times in the adjuvant are shown in a semi-logarithmic plot in Figure 6.1. The enzyme activity, which is proportional to the amount of native  $\beta$ -lactamase, was decreasing dramatically over time in Freund's adjuvant. The half-life time of the enzyme activity estimated from this data was about 21 minutes. (It was also noticed that the presence of 5% glycerol in the water phase when mixing the adjuvant would protect the enzyme activity in the adjuvant for at least 24 hours although the enzyme activity was not carefully quantified in this case).

### **Extraction methods and enzyme activity**

The enzyme activity of  $\beta$ -lactamase decreased over time in Freund's adjuvant but the data in Figure 6.1 do not extrapolate back to 1.0 in the normalised plot and the ether's impact on the  $\beta$ -lactamase was therefore investigated.  $\beta$ -Lactamase in PBS was mixed with ether and vortexed for two minutes, the maximum time  $\beta$ -lactamase was expected

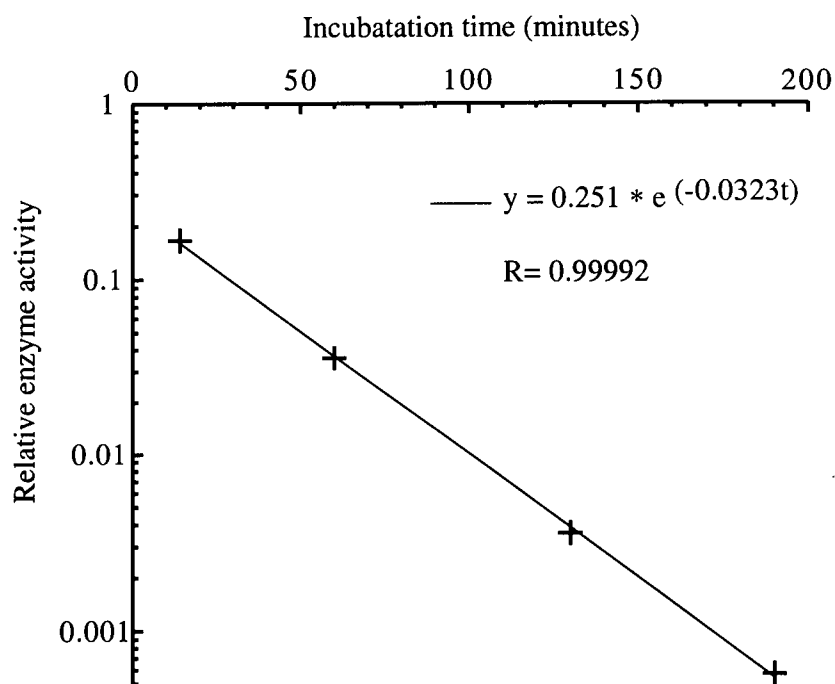


Figure 6.1:  $\beta$ -Lactamase in PBS was emulsified in Freund's adjuvant. The water phase was extracted after different incubation times in the adjuvant and the enzyme activity in the water phase was measured with the chromogenic  $\beta$ -lactamase substrate Nitrocefin. The value 1.0 represents the enzyme activity of  $\beta$ -lactamase which has not been in Freund's adjuvant. The fitted line is given by the equation  $f = 0.251 \cdot e^{-0.0323 \text{ min}^{-1} \cdot t}$ . The R-value of the fitted line is 0.9992.

to be in contact with ether in the extraction method. No reduction in enzyme activity was detected (data not shown). PBS emulsified in Freund's incomplete adjuvant was then separated from the oil phase by ether and mixed with  $\beta$ -lactamase to evaluate if any co-extracted substances could inhibit the enzyme activity. The enzyme activity in the presence of extracted buffer was about 70% of the activity measured in "fresh" PBS and did not change over a time span of two hours (data not shown).

### 6.2.3 Assaying denaturation by proteolysis of $\beta$ -lactamase

The adjuvant's impact on the state of  $\beta$ -lactamase was also examined through susceptibility to proteolysis. Native  $\beta$ -lactamase was resistant to the proteolytic activity of trypsin for at least two hours at room temperature when mixed in a ratio 1:100 trypsin to  $\beta$ -lactamase (control in Figure 6.2).  $\beta$ -Lactamase has several recognition sites for trypsin in the linear sequence and denature  $\beta$ -lactamase was therefore expected to be cleaved by trypsin.  $\beta$ -Lactamase was emulsified in Freund's adjuvant in the presence of

trypsin and the water phase was after different incubation times separated from the adjuvant by adding ether. The trypsin activity was immediately stopped with PEFA-block followed by loading of the samples mixed with sodium dodecyl sulphate (SDS) on a gel. The results are shown in Figure 6.2a. Cleaved fragments of  $\beta$ -lactamase appeared after a short incubation time in the adjuvant.  $\beta$ -Lactamase in native form was resistant to the same amount of trypsin for at least two hours as shown in the control.

$\beta$ -Lactamase was also emulsified the same way without trypsin present and after separation from the adjuvant loaded on the gel shown in Figure 6.2b. No cleaved product of  $\beta$ -lactamase, as expected, were visible in this gel. Bands corresponding to the size of two or more  $\beta$ -lactamase molecules were present in this gel. These bands were also present if hexane or oil were used for extraction of the protein (data not shown), which means that the cross-linking of the protein was not due to the ether. The cross-linked  $\beta$ -lactamase was absent in the samples with trypsin (Figure 6.2a). This suggests that the cross-linked protein comprised denatured protein which was proteolysed in the presence of trypsin.

The extraction of  $\beta$ -lactamase from Freund's adjuvant without any trypsin present yielded large precipitates with  $\beta$ -lactamase (see lane next to control in Figure 6.2b). The precipitation may have been a result of high concentration of denatured protein in the emulsions. The large precipitates were also observed if the antigen was extracted by hexane or oil, which suggest that they were formed independently of the extraction method. Little precipitates were seen if trypsin was co-incubated with the  $\beta$ -lactamase in the adjuvant, which may be due inhibition of the precipitation by proteolysis of the denatured protein.

The samples with  $\beta$ -lactamase which was cross-linked were also incubated in 8M urea and 2% SDS at 70°C for 20 minutes. Dithiothreitol (DTT) was added to a concentration of 50mM and the samples were then incubated 10 minutes at room temperature and 70°C for 15 minutes. Running the samples through a gel (Figure 6.3) showed that

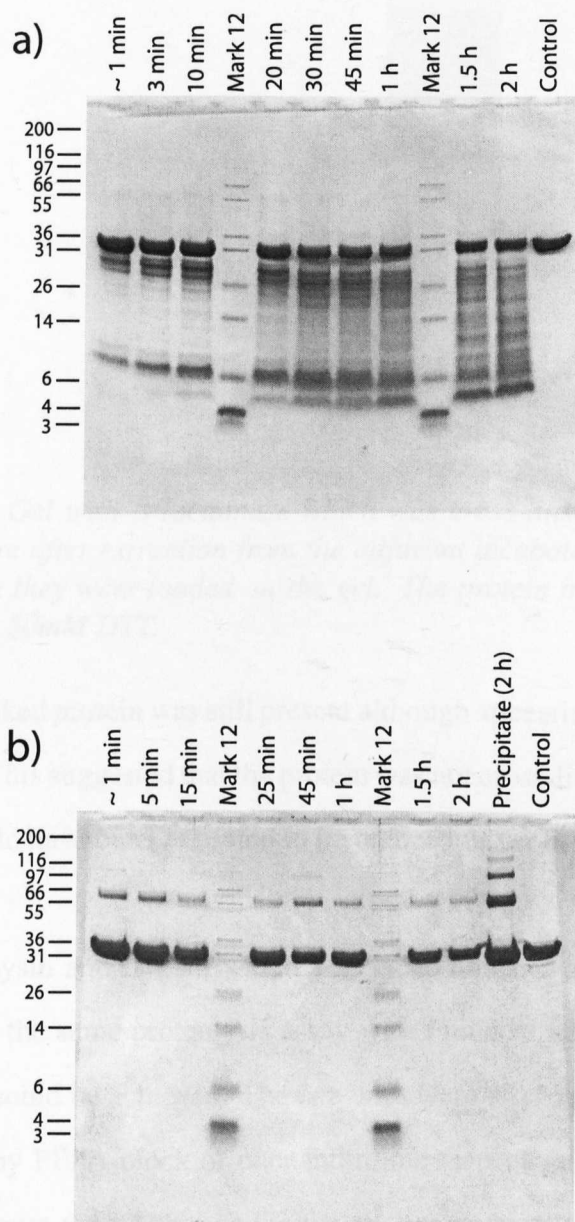


Figure 6.2: a)  $\beta$ -lactamase was mixed with small amounts of trypsin and emulsified in Freund's adjuvant. Aliquots of the emulsion were incubated for different times before the protein was extracted. The trypsin was inactivated and the samples were loaded on a gel with SDS. The control was  $\beta$ -lactamase with the same amount of trypsin for two hours, but had not been in Freund's adjuvant. b)  $\beta$ -Lactamase was emulsified and extracted the same way as above, but there was no trypsin present. The extracted samples also contained precipitates that were separately dissolved and loaded on the gel (next to the control). The samples in image b were incubated with DTT for a short time and some unreduced protein (band 2) can be seen further down than the reduced form of the protein (band 1). The control was  $\beta$ -lactamase incubated with the same amount of trypsin for two hours, but had not been in Freund's adjuvant.

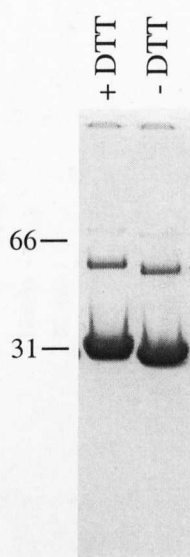


Figure 6.3: Gel with  $\beta$ -lactamase which was cross-linked in Freund's adjuvant. The samples were after extraction from the adjuvant incubated in 8M urea and 2% SDS at 70°C before they were loaded on the gel. The protein in the left lane was in addition treated with 50mM DTT.

the cross-linked protein was still present although appearing a slightly different positions on the gel. This suggested that the protein was not cross-linked through disulphide bonds as they would have been expected to be reduced under these conditions.

Thermolysin and trypsin which was cross-linked with hydrophilic polymers were also used in the same proteolysis assay with Freund's adjuvant to test if higher rate of proteolysis could be achieved. The enzymes were after incubation in Freund's adjuvant inactivated by PEFA-block or phenanthroline respectively. The proteolysis results are shown in Figure 6.4. Although the figure suggest more extensive proteolysis of the  $\beta$ -lactamase, especially with thermolysin, some full-length protein was still seen in this gel after two hours incubation in the adjuvant.

#### 6.2.4 Assaying denaturation of other antigens by proteolysis

$\beta$ -Lactamase was denatured by Freund's adjuvant as shown in this chapter. The impact of Freund's adjuvant on the state of some other model antigens used in earlier epitope studies was also evaluated. Proteolysis experiments with trypsin as described in the previous sections were carried out with bovine serum albumin (BSA), hen egg lysozyme

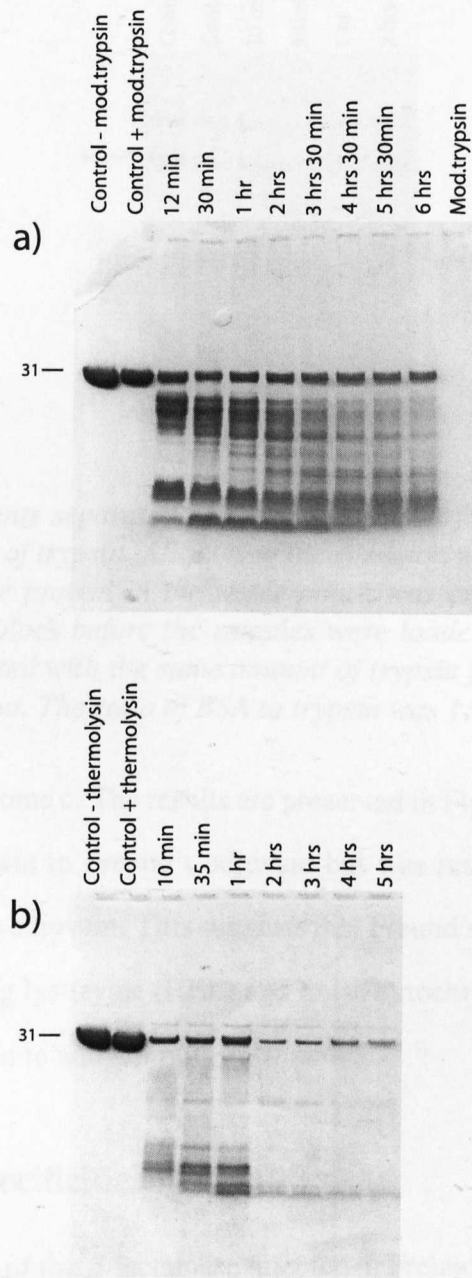


Figure 6.4:  $\beta$ -Lactamase fragments separated on a gel with SDS after incubation in Freund's adjuvant in the presence of cross-linked trypsin. The trypsin was cross-linked with hydrophilic polymers. Aliquots of the emulsion were incubated for different time intervals before the protein in the water phase was extracted. The trypsin was inactivated before the samples were loaded on gels with SDS. Control:  $\beta$ -lactamase was incubated with or without the same amount of trypsin for six hours, but had not been in Freund's adjuvant. The ratio of  $\beta$ -lactamase to modified trypsin was 1:10 (w/w) b) The same experiment as in image (a) but with thermolysin instead of cross-linked trypsin. The ratio of  $\beta$ -lactamase to thermolysin was 1:50 (w/w)

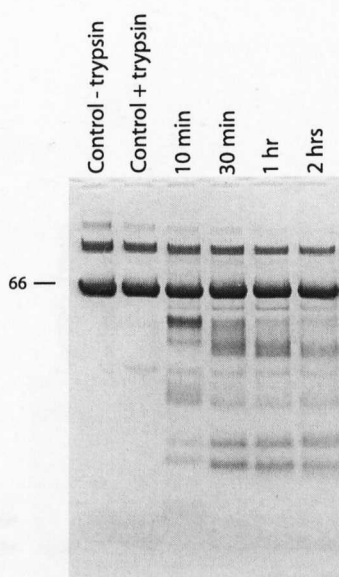


Figure 6.5: *BSA fragments separated on a gel with SDS after incubation in Freund's adjuvant in the presence of trypsin. Aliquots of the emulsion were incubated for different time intervals before the protein in the water phase was extracted. The trypsin was inactivated with PEFA-block before the samples were loaded on a gel with SDS. The control was BSA incubated with the same amount of trypsin for two hours, but had not been in Freund's adjuvant. The ratio of BSA to trypsin was 1:100 (w/w).*

(HEL) and horse cytochrome c. The results are presented in Figure 6.5 and 6.6. The BSA was proteolysed by trypsin in Freund's adjuvant but was resistant to the same trypsin concentration outside the adjuvant. This suggests that Freund's adjuvant denatured BSA. No proteolysis of hen egg lysozyme (HEL) and horse cytochrome c in the adjuvant was observed even at a trypsin to antigen ratio 1:10 (w/w).

### 6.2.5 Antibody specificities in sera

It was obvious that some of the  $\beta$ -lactamase injected in Freund's was denatured and Freund's adjuvant's influence on the antibody specificities in the antiserum was therefore analysed. The analysis included sera from the two rabbits (rabbits 1 and 2) immunised with  $\beta$ -lactamase in Freund's adjuvant and the two rabbits (rabbits 3 and 4) immunised with  $\beta$ -lactamase in PBS. Only the IgG fraction, the same as used in the epitope mapping, was investigated. There was not enough sera from the mice immunised with immunised with  $\beta$ -lactamase in Freund's adjuvant for the subsequent analyses and the two mice immunised with  $\beta$ -lactamase in PBS did not produce detectable amounts of IgG.



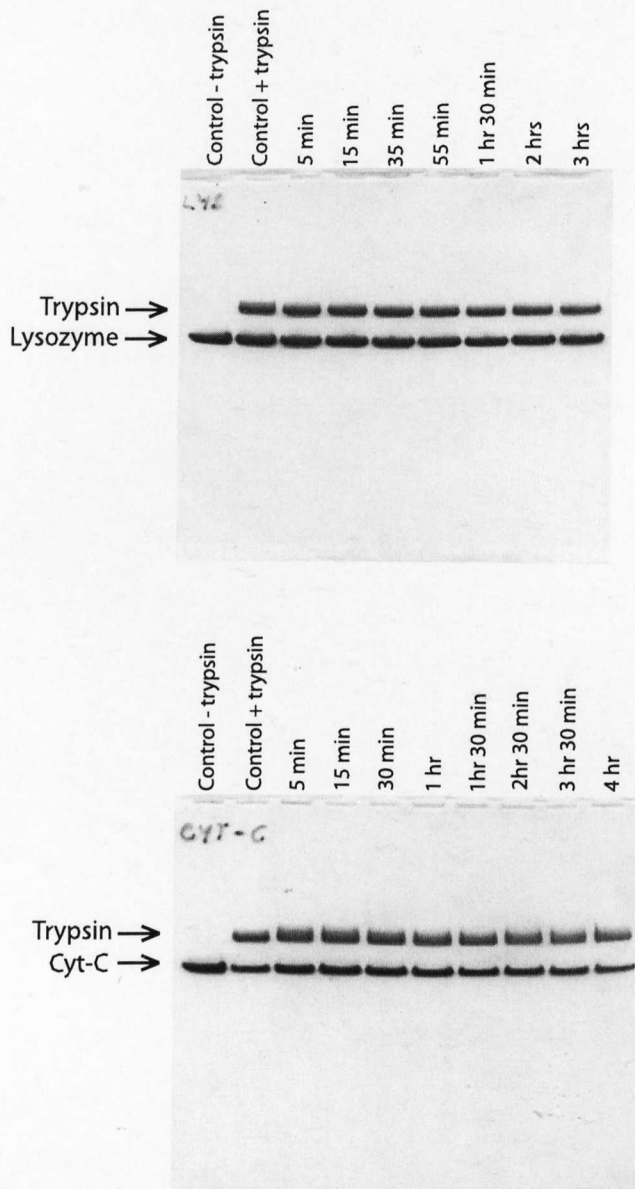


Figure 6.6: Hen egg lysozyme (HEL) (a) and cytochrome c (b) fragments separated on a gel with SDS after incubation in Freund's adjuvant in the presence of trypsin. Aliquots of the emulsion were incubated for different time intervals before the protein in the water phase was extracted. The trypsin was inactivated before the samples were loaded on a gel with SDS. The control was lysozyme/cytochrome c incubated with or without the same amount of trypsin for three hours, but had not been in Freund's adjuvant. The ratio of BSA to trypsin was 1:10 (w/w).



### **Antibody binding native or denatured protein**

The rabbit antibodies were first analysed for binding to native or denatured  $\beta$ -lactamase in ELISA. The results are shown in Figure 6.7 (See also Figure A.1 on page 146). The biotinylated  $\beta$ -lactamase (308C) was immobilised on streptavidin coated ELISA plates and was denatured on the plates by reducing the internal disulphide bond by DTT and carboxymethylating the two cysteines. The biotinylation reagent was in this case a maleimide activated biotin-linker as the linker used in the mapping assay could easily be reduced by DTT. (The absence of native antigen was confirmed by adding the chromogenic enzyme substrate Nitrocefin and the presence of similar amounts of native and denatured antigen on the plates was confirmed by detecting the C-terminal FLAG-tag (peptide tag) on the  $\beta$ -lactamase with a secondary antibody). The serum antibodies from the two rabbits (rabbits 1 and 2) immunised with  $\beta$ -lactamase in Freund's adjuvant bound both native and denatured protein. The sera from two rabbits (rabbit 3 and 4) immunised with antigen in PBS bound strongly to native but only weakly to denatured protein. Serum from rabbit 4 showed hardly any signal on denatured protein.

The same sera were also analysed in western blots. The  $\beta$ -lactamase was partly digested with trypsin, cyanogen bromide or Lys-C before fractionated on a gel with SDS and then transferred to nitro-cellulose membranes. Serum antibodies were incubated with the membranes followed by detection of antibody binding with secondary antibodies conjugated to an enzyme. The results are shown in Figure 6.8. The serum antibodies from rabbits 1 and 2, immunised with  $\beta$ -lactamase in Freund's adjuvant, bound strongly to the proteolytic fragments. The serum antibodies from rabbit 3 bound some fragments although fewer and with less intense signal than the previous rabbits. The serum antibodies from rabbit 4 did not recognise the fragments of  $\beta$ -lactamase, only full or nearly full-size protein was recognised by antibodies in this serum. Adding the chromogenic substrate Nitrocefin to the membrane demonstrated that the two bands on the nitro-cellulose membrane recognised by these antibodies contained native protein

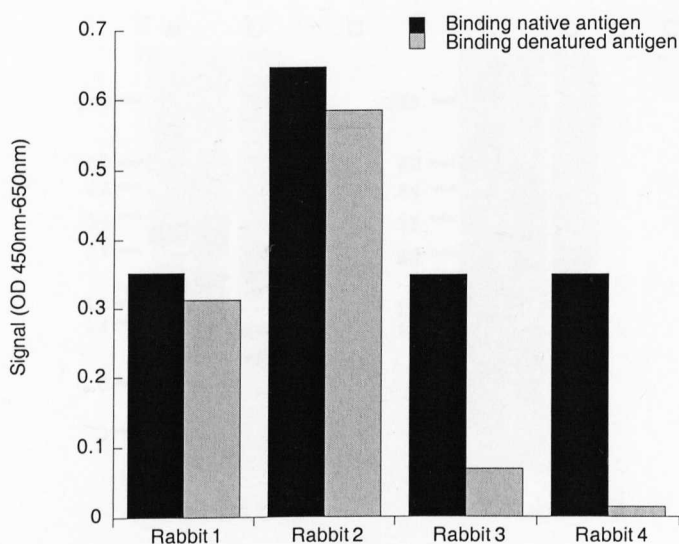


Figure 6.7: The ELISA results with sera antibodies from rabbits immunised with antigen in Freund's adjuvant (rabbits 1 and 2) or in PBS (rabbits 3 and 4). The antibodies were assayed for their ability to bind native or denatured  $\beta$ -lactamase. Only the IgG fractions of the antibodies were analysed. The biotinylated  $\beta$ -lactamase was captured on streptavidin coated ELISA plates. The protein was denatured by reducing an internal disulphide bond and carboxymethylating the cysteines. The serum taken 10 days after the second antigen boost was diluted 1:25,000.

(picture not shown). These two bands were probably  $\beta$ -lactamase that had migrated on the gel in with the internal disulphide bond in intact or reduced form.

### Native-specific and denatured-specific antibodies

Antibodies in sera can be divided into three groups as described in the main introduction; denatured-specific, native-specific and cross-reactive antibodies of which the latter group bind both native and denatured antigen. Cross-reactive antibodies could therefore have contributed to binding on both native and denatured protein in the assays described in the previous sections. The serum from rabbit 1 was therefore also analysed for the presence of denatured-specific and native-specific antibodies separately. Serum aliquots were first depleted for antibodies binding native or denatured protein and the remaining antibodies were then assayed for binding the same or the other form. The depletion was carried out by repeatedly moving the serum aliquots between ELISA plate wells with either denatured or native antigen. The  $\beta$ -lactamase was biotinylated and captured on streptavidin coated ELISA plates (native protein) or coated directly on ELISA plates followed by drying the plates for one hour (denatured protein). (The latter had no de-

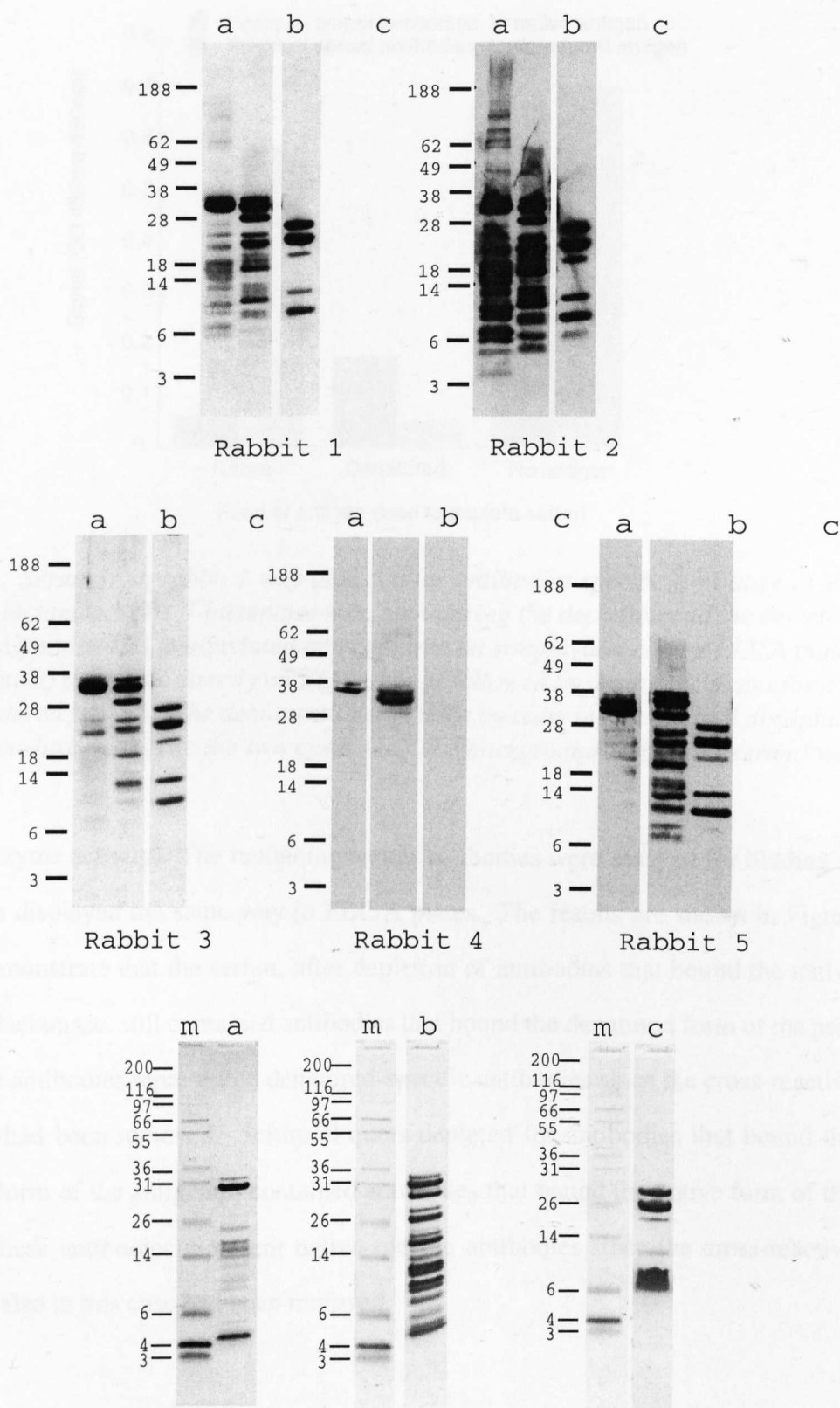


Figure 6.8: Serum antibodies were assayed for their ability to bind fragmented  $\beta$ -lactamase in western blots (the five upper membranes). The bands recognised by serum from rabbit 4 were shown to include native protein by adding the substrate Nitrocefin to the nitro-cellulose membrane (picture not shown). Rabbit 5 is described later in this chapter. The lower gels show Coomassie stained gels with the same fragments. The  $\beta$ -lactamase was fragmented by trypsin (a), cyanogen bromide (b) and Lys-C (c). Lane m contained protein standards of known sizes.

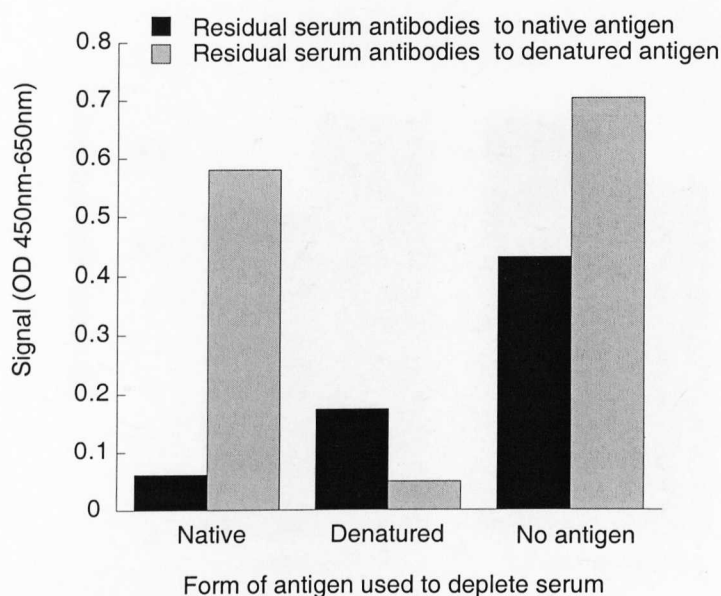


Figure 6.9: Serum from rabbit 1 was depleted for antibodies specific for native or denatured  $\beta$ -lactamase. The  $\beta$ -lactamase was, both during the depletion and the detection of remaining antibodies, biotinylated and captured on streptavidin coated ELISA plates (native protein) or coated directly on ELISA plates followed by drying the plates for one hour (denatured protein). The denaturation was done by reducing an internal disulphide bond and carboxymethylate the two cysteines. The background signal (no serum) was 0.017.

tectable enzyme activity). The remaining serum antibodies were assayed for binding to the antigen displayed the same way in ELISA plates. The results are shown in Figure 6.9 and demonstrate that the serum, after depletion of antibodies that bound the native form of  $\beta$ -lactamase, still contained antibodies that bound the denatured form of the protein. These antibodies represented denatured-specific antibodies since the cross-reactive antibodies had been removed. Serum aliquots depleted for antibodies that bound the denatured form of the antigen in contained antibodies that bound the native form of the protein. These antibodies represent native-specific antibodies since the cross-reactive antibodies also in this case had been removed.

### Cross-reactive antibodies

The serum from rabbit 1 was further assayed for presence of cross-reactive antibodies. Serum antibodies were affinity purified on native or denatured (carboxymethylated)  $\beta$ -lactamase (308C). These antibodies were then examined for their ability to bind de-

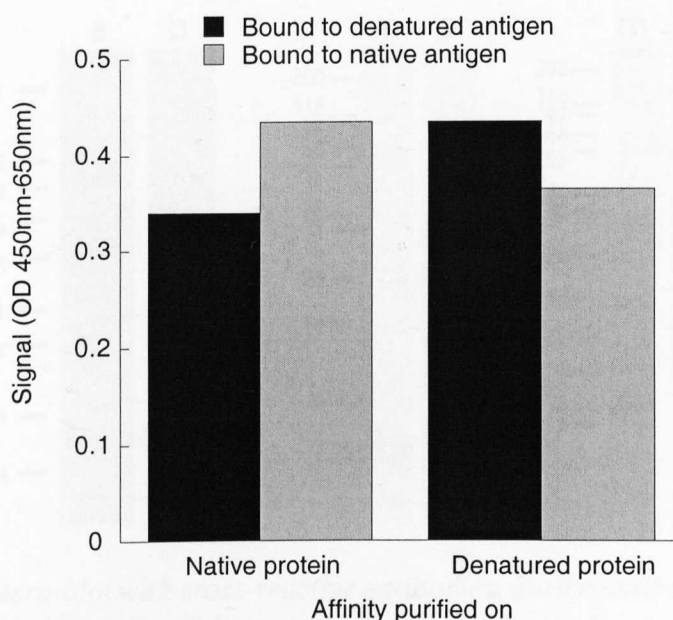


Figure 6.10: *Cross-reactive serum antibodies from rabbit 1. The antibodies were affinity purified on native or denatured  $\beta$ -lactamase and then assayed for binding to denatured or native protein respectively. The  $\beta$ -lactamase was biotinylated and captured on streptavidin beads during the affinity purification and on streptavidin coated ELISA plates during the detection. The protein was denatured by reducing an internal disulphide bond and carboxymethylate the cysteines. The same amount of serum and beads were used for all the samples. The background signal was 0.011.*

natured or native protein respectively which was presented on ELISA plates as described earlier. The results are shown in Figure 6.10. The serum contained antibodies that both could be affinity purified on native protein and bound denatured protein and vice versa. The presence of these cross-reactive antibodies were also confirmed by western-blot as shown in Figure 6.11. Serum antibodies affinity purified on native protein were assayed for binding to  $\beta$ -lactamase fragments on a nitro-cellulose membrane. The serum obviously contained antibodies that could bind both native protein in the affinity purification and shorter fragments of the protein as shown on the nitro-cellulose membranes in the western blots.

### 6.2.6 Mapping of cross-reactive antibodies

The epitopes of the cross-reactive antibodies were mapped separately as they were expected to play an important role in the epitope distribution as discussed later. The map-

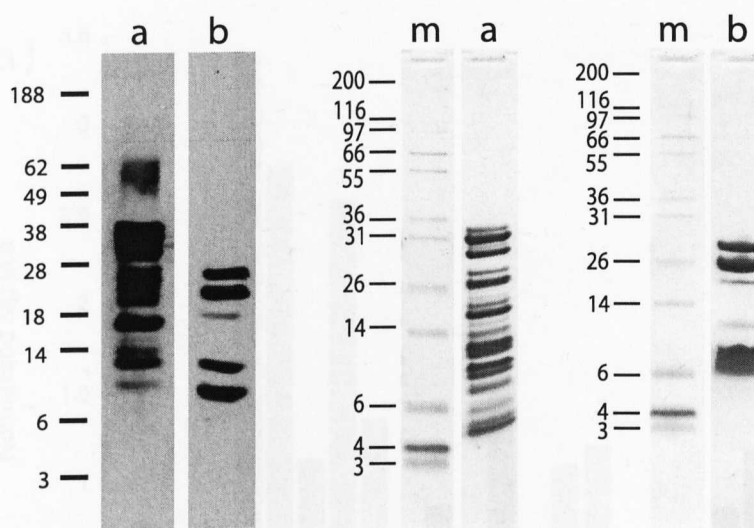


Figure 6.11: Western-blot with cross-reactive antibodies. Serum antibodies from rabbit 1 were affinity purified on native  $\beta$ -lactamase. The eluted antibodies were then allowed to bind fragments of  $\beta$ -lactamase on a nitro-cellulose membrane shown in the two lanes on the left. Bound antibodies were detected with anti-rabbit antibodies conjugated to HRP. The two lanes on in the middle and the two lanes on the right are Coomassie stained gels with the same protein fragments as used in the on the membrane. Lanes a and b were fragments of  $\beta$ -lactamase digested with cyanogen bromide or Lys-C respectively. Lane m contained protein standards of known sizes.

ping assay was essentially carried out as described in chapter 3 with the exception that the depletion step (isolation of antibodies specific for each CTR) was followed by affinity purification of antibodies on native protein (biotinylated 308C) and then analysis of binding of these antibodies to denatured  $\beta$ -lactamase. (The protein was denatured by coating directly on ELISA plates followed by drying the plates for one hour. This  $\beta$ -lactamase had no detectable enzyme activity). The result from this epitope mapping are shown in Figure 6.12 together with the mapping results for the cross-reactive plus the native-specific antibodies described in chapter 4. The two epitope patterns in this figure did both show the highest signal in the loop region D85 to V119. The difference in the signals on CTR-271 and CTR-281 in plot b) and a) may well have been due native-specific antibodies as these were removed in plot a). Differences in antibody binding to  $\beta$ -lactamase captured on beads during the affinity purification or plates during detection, as discussed in chapter 3, may also count for some of the variations in the epitope patterns between these two plots.



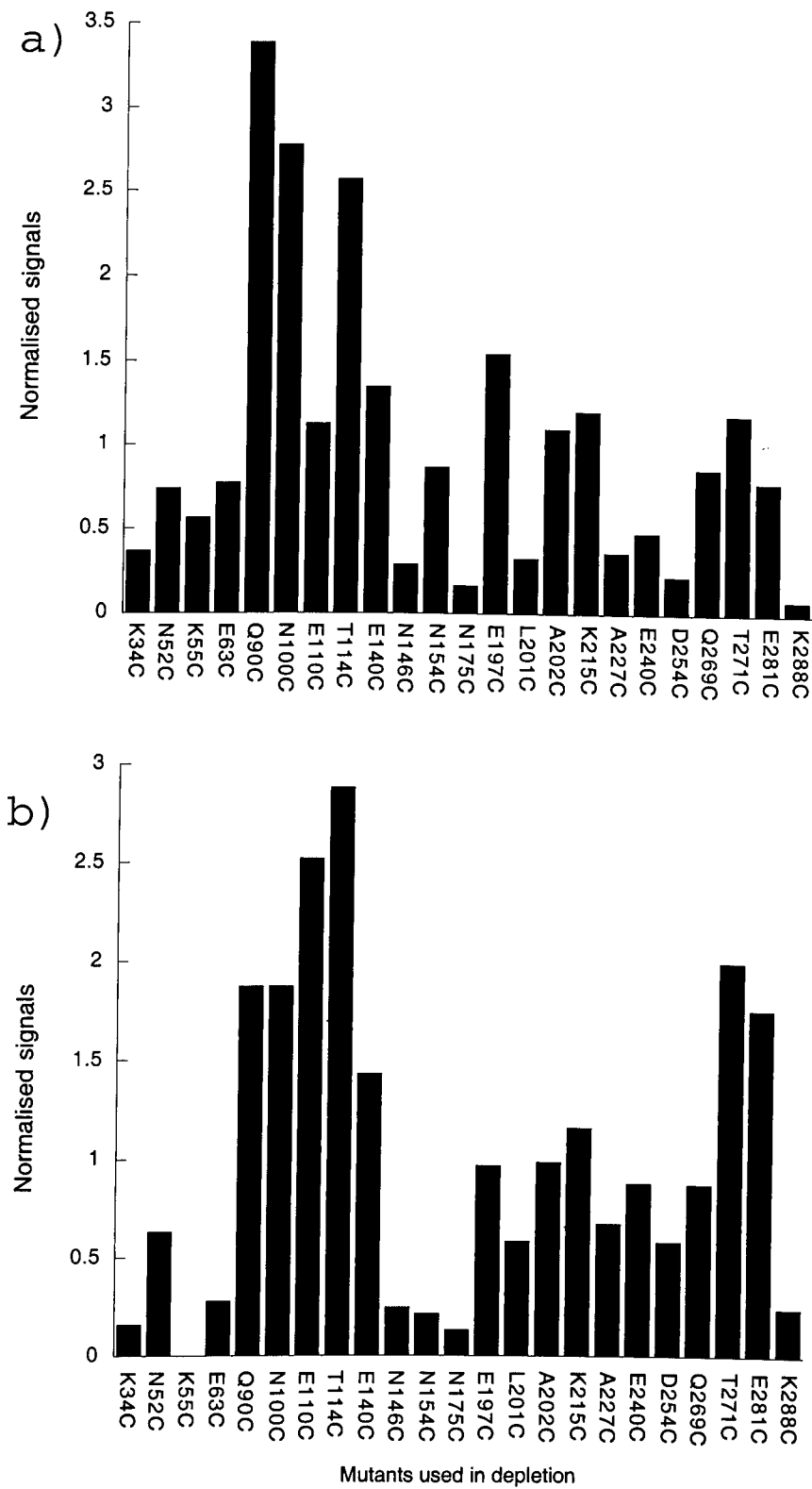


Figure 6.12: The distribution of cross-reactive antibodies from rabbit 1 on the surface of  $\beta$ -lactamase (a) and cross-reactive plus native-specific antibodies (b). The latter plot is the same as in Figure 4.1a. Mapping of the cross-reactive antibodies alone was done by isolating of serum antibodies against each CTRs, as described for the main assay in chapter 3, followed by affinity purification on native  $\beta$ -lactamase and detection of binding on denatured  $\beta$ -lactamase. The  $\beta$ -lactamase had been denatured by coating directly on ELISA plates which were then dried for one hour. Cross-reactive and native-specific antibodies were mapped together as described in chapter 3. The serum concentrations were in 1:10,000 in both experiments.

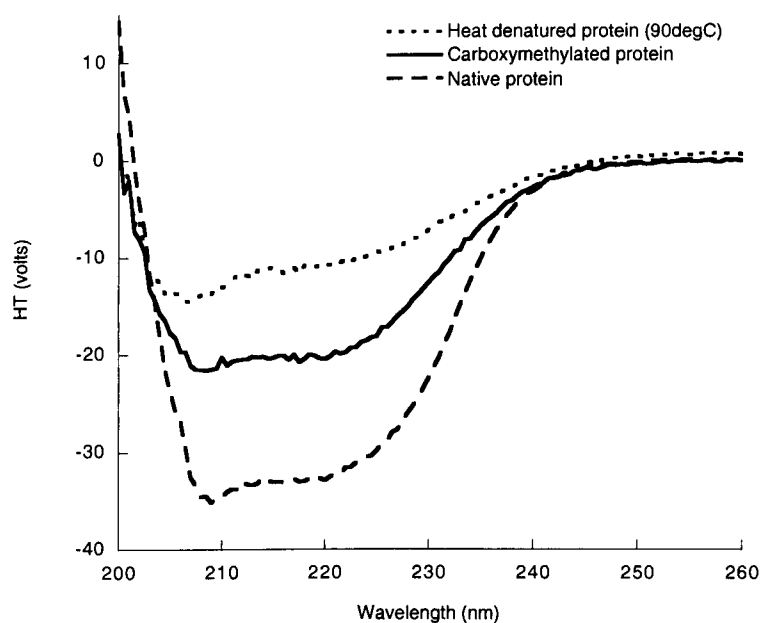


Figure 6.13: CD scans of native, carboxymethylated and heat denatured  $\beta$ -lactamase.

### 6.2.7 Immunisation with denatured protein

One question arising from this work was whether immunisation with denatured protein can give rise to antibodies recognising native protein. One rabbit (rabbit 5) was therefore immunised with irreversibly denatured  $\beta$ -lactamase. The  $\beta$ -lactamase was denatured by reducing the internal disulphide bond and carboxymethylating the two cysteines. Circular dichroism (CD) spectroscopy was used to examine if the carboxymethylated protein had any residual secondary structure and the results are shown in Figure 6.13. Reversible thermal denaturation was not obtained for the carboxymethylated  $\beta$ -lactamase and the folding energy of this protein was therefore not be calculated. The CD curves indicate that the carboxymethylated protein has more secondary structure than the heat denatured protein but less than the native protein. The carboxymethylated protein was still believed to be denatured as it had an enzyme activity of about 1:10,000 compared to the native protein. The carboxymethylated  $\beta$ -lactamase was also highly susceptible to proteolysis in contrast to native  $\beta$ -lactamase which was resistant to proteolysis under the same conditions as shown in Figure 6.14.

The carboxymethylated  $\beta$ -lactamase was injected with Freund's adjuvant. The binding of the serum antibodies from the different test-bleeds to native or denatured  $\beta$ -



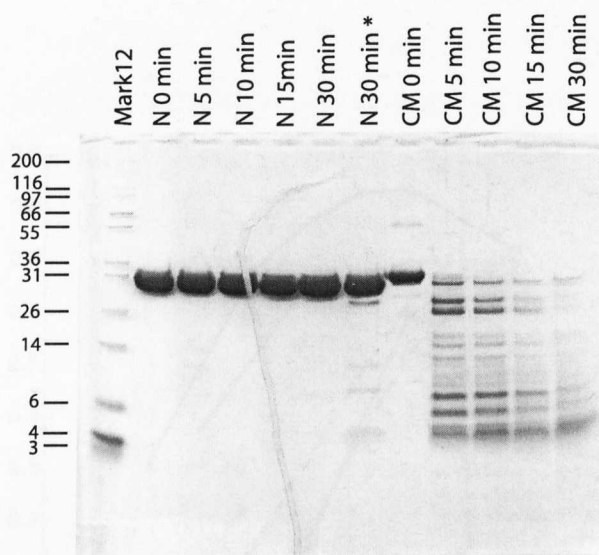


Figure 6.14: Native and denatured (carboxymethylated) protein treated with trypsin. The ratio of  $\beta$ -lactamase to trypsin was 1:100 (w/w) (except lane\* which was 1:25 (w/w)). The samples were run on a gel with SDS. The native samples contain 10 $\mu$ g protein and denatured samples contain 4.7 $\mu$ g protein.

lactamase are shown in Figure 6.15. The serum antibodies did recognise the native biotinylated  $\beta$ -lactamase (308C) on a streptavidin coated ELISA plate. This means that the carboxymethylated  $\beta$ -lactamase, which was not in native conformation, induced the production of antibodies that nevertheless recognised the native protein. The ability of serum antibodies from rabbit 5 to capture  $\beta$ -lactamase from solution was poor as shown in Table 6.1. The difference in antibody binding to antigen in solution or immobilised on a solid surface was probably due to low affinities as binding to the antigen captured on solid the phase is likely to have been enhanced by the avidity of IgG.

### 6.2.8 Mapping anti CM- $\beta$ -lactamase antibodies

The epitope distribution in the sera from rabbit 5, immunised with carboxymethylated  $\beta$ -lactamase, was also examined by the mapping method described in chapter 3. The results are shown in Figure 6.16. The epitope pattern revealed (after subtracting a high signals on "Masked" as discussed bellow) two surface regions dominating the epitope distribution on the native  $\beta$ -lactamase. These are located around the CTRs 90, 110 and 114 and the CTRs 197, 201 and 202.

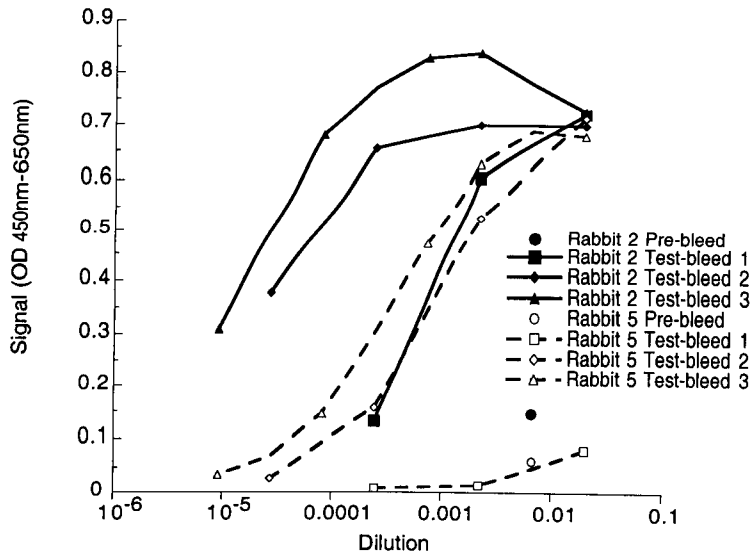


Figure 6.15: Serum from rabbits 2 and 5 were tested for the ability to bind native  $\beta$ -lactamase. Biotinylated  $\beta$ -lactamase was immobilised on streptavidin coated ELISA plates. Rabbit 2 and 5 were immunised with  $\beta$ -lactamase or carboxymethylated  $\beta$ -lactamase in Freund's adjuvant respectively.

Table 6.1: Serum antibodies displayed on ELISA plates coated with anti-rabbit antibodies were used to immobilise  $\beta$ -lactamase added in solution. The enzyme activity of  $\beta$ -lactamase was used to detect bound protein (second column). The relative amount of serum antibodies on the plate was detected by a secondary antibody (last column) to allow comparison of the signal in column two.

Rabbit	Detected $\beta$ -lactamase [mOD/min]	Detected rabbit antibodies [OD]
Rabbit 1	14.35	0.576
Rabbit 2	22.36	0.677
Rabbit 3	25.75	0.614
Rabbit 4	23.98	0.652
Rabbit 5	0.66	0.538
Background	0.19	0.011

The epitope mapping of serum from rabbit 5 gave high signals on "Masked" (Figure 6.16). It is assumed that this was due to large amounts of antibodies with low affinity for the native protein. The fact that for some CTRs, the signal "Masked" is greater than "Exposed" appears to be due to the presence of denatured antigen in these CTRs (mutants) (as established by the monoclonal antibodies in chapter 2).

### 6.3 Discussion

The results in this chapter show clearly that  $\beta$ -lactamase was denatured by Freund's adjuvant. The half-life time of the native  $\beta$ -lactamase in Freund's adjuvant was about 21 minutes. The fitted line in Figure 6.1 does not extrapolate back to 1.0 in the normalised plot, but starts at 0.25. The initial loss of 75% may have been due to denaturation of the protein during the emulsification process or denaturation during the extraction process of which the latter would be an artefact in the assay. But none of these interpretations would change the slope of the line in Figure 6.1. Ether which was used in the extraction did not denature  $\beta$ -lactamase in PBS and co-extracted chemicals from Freund's adjuvant reduced the activity by 30% did only account for a smaller part of the initial loss in enzyme activity.

The animals were injected typically one hour after the antigen was emulsified in the adjuvant. With the half-life time of the native  $\beta$ -lactamase in the adjuvant of 21 minutes, approximately 14% would have remained native after one hour, assuming that the initial 75% drop observed in the enzyme activity was due to an artefact of the extraction process used in the analysis of the protein. If the initial drop in contrast did take place in the emulsification process, then only 3.5% of the antigen would have remained native when injected into the animals. In both cases the vast majority of the  $\beta$ -lactamase would have been denatured before injected into the animals. Freund's adjuvant is in addition very stable in animals [Herbert, 1968] and the antigen would therefore have had even more time to denature in the adjuvant after injection.

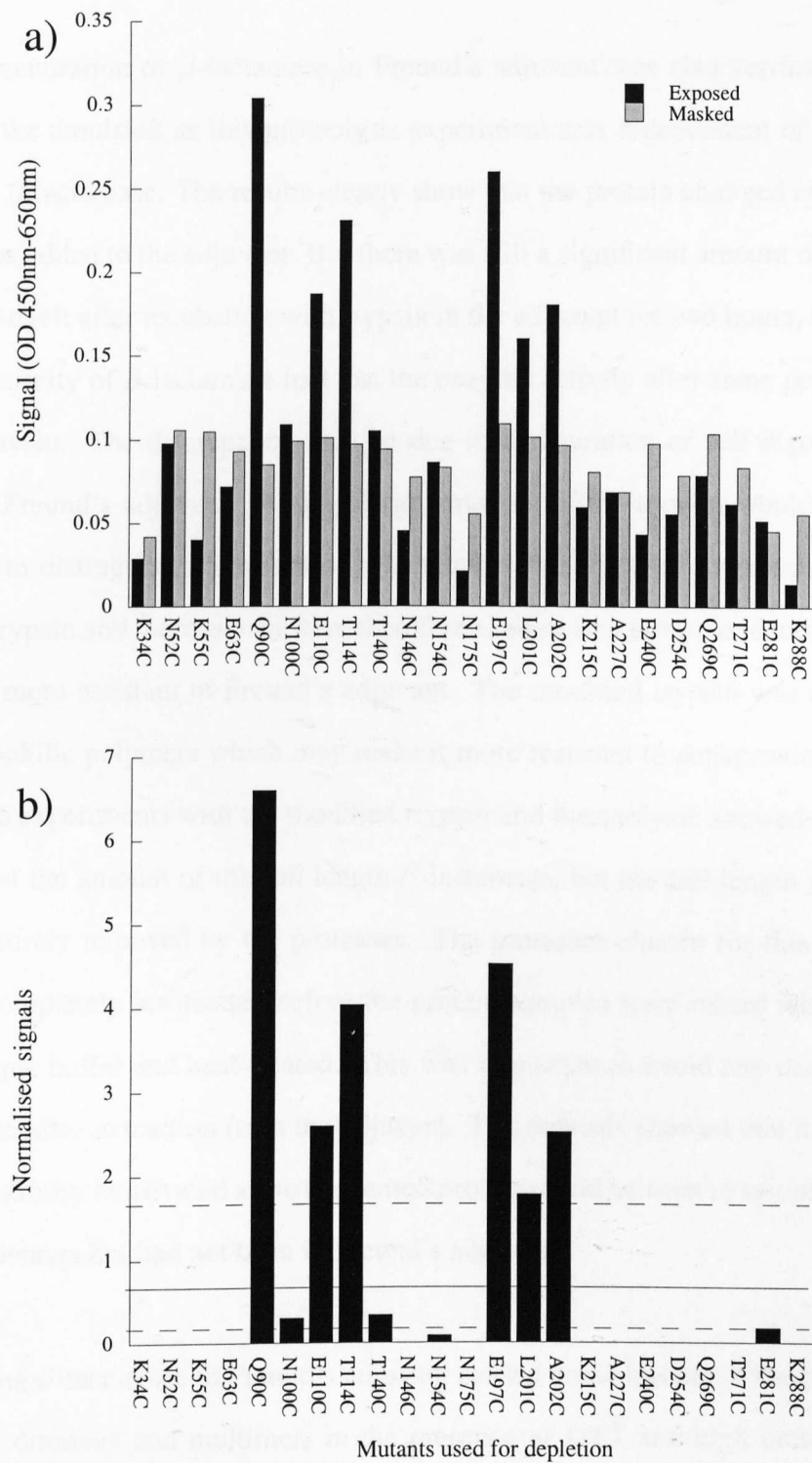


Figure 6.16: Epitope distribution for rabbit 5. a) Signals on "Exposed" and "Masked" for each CTR. b) The relative distribution of serum antibodies to different surface locations for rabbit 5. The signals are normalised to give an average of 1. The signals on "Masked" are subtracted and negative values are set to zero. The average value is indicated with a full line. The dotted lines indicate half the average signal and double the average signal. The sera concentrations in the mapping assay was 1:30,000. The rabbit was primarily injected with carboxymethylated  $\beta$ -lactamase in FCA followed by carboxymethylated  $\beta$ -lactamase in FIA in first and second boost.

The denaturation of  $\beta$ -lactamase in Freund's adjuvant was also verified by adding trypsin to the emulsion as this proteolysis experiment was independent of the enzyme activity of  $\beta$ -lactamase. The results clearly show that the protein changed conformation when it was added to the adjuvant. But there was still a significant amount of full-length  $\beta$ -lactamase left after incubation with trypsin in the adjuvant for two hours, even though the vast majority of  $\beta$ -lactamase had lost the enzyme activity after same period of time in the adjuvant. The discrepancy may be due to denaturation or self-digestion of the trypsin in Freund's adjuvant. Adding large amounts of the trypsin would have made it difficult to distinguish fragments of  $\beta$ -lactamase from cleaved fragments of trypsin. Modified trypsin and thermolysin were therefore used in an attempt to identify proteases which are more resistant to Freund's adjuvant. The modified trypsin was cross-linked with hydrophilic polymers which may make it more resistant to denaturation in the adjuvant. The experiments with the modified trypsin and thermolysin showed a fast initial reduction of the amount of the full length  $\beta$ -lactamase, but the full length  $\beta$ -lactamase was not entirely removed by the proteases. The proteases chosen for this experiment could be completely inactivated before the protein samples were mixed with the denaturing sample buffer and heat-treated. This was important to avoid any degradation of  $\beta$ -lactamase after extraction from the adjuvant. The controls showed that the proteases were successfully inactivated as no fragmented protein could be seen in samples that contain the proteases but had not been in Freund's adjuvant.

Exposing  $\beta$ -lactamase to Freund's adjuvant caused cross-linking of the protein. The stability of dimmers and multimers in the presence of DTT and high urea concentrations indicated that they were not cross-linked through disulphide bonds, but through other kinds of covalent linkage. Treatment with DTT slightly reduced the mobility of the protein on gels with SDS. This was probably due to reduction of the intact intramolecular disulphide bond also present in the native monomeric protein. This suggests that the cysteines were not involved in the covalent cross-linking. The absence of the cross-linked protein in the samples that had been co-incubated with trypsin in the adju-

vant indicated that the multimers probably were denatured protein. The multimerisation was not dependent on the extraction method used which implies that it was an effect of the adjuvant. The cross-linking of the antigen is thought have been caused by peroxides in the adjuvant, but no analysis was carried out to test this hypothesis.

The  $\beta$ -lactamase, which was expressed and purified from *E. coli*, was probably native as discussed in chapter 2, but immunisation with this antigen in Freund's adjuvant gave sera with antibodies that bound strongly to the denatured protein and some of the antibodies did only bind the linear form of the protein. This is consistent with the results which showed that the adjuvant denatured the antigen. The rabbits immunised with antigen in PBS produced only low titres of antibodies recognising denatured protein as expected if the injected protein was native.

Both carboxymethylated and fragmented protein were here used as examples of denatured protein. The results did therefore give strong evidence for lack of cross-reactive or denatured-specific antibodies in the serum from rabbit 4 as this serum showed no binding to the carboxymethylated protein in ELISAs or to the fragments in western blots. The only bands in the western blots that bound antibodies in this serum, had the apparent size of full-length protein and were shown to contain native protein. No binding of denatured protein by serum from rabbit 4 was therefore proved, and the lack of binding to the smaller fragments suggests that this sera did not bind denatured  $\beta$ -lactamase at all. The serum from rabbit 3 did to some extent recognise some of the  $\beta$ -lactamase fragments in the western blots. This did not contradict the ELISA results as even weak binding was expected to give signal in the western blots due to the high density of homogeneous antigen fragments in each band.

The rabbits immunised with Freund's adjuvant produced cross-reactive and denatured-specific antibodies as expected since the adjuvant contained large amount of denatured antigen. An important question was then if the denatured protein could change the epitope distribution detected on the native protein. If this was the case then one may expect

that the cross-reactive antibodies did play an important role in the epitope response as described in more detail later. These antibodies did dominantly bind the same flexible loop region, D85 to V119, which also dominated the antibody responses in the animal immunised with  $\beta$ -lactamase in Freund's adjuvant.

Immunisation with chemically denatured protein showed that denatured protein in the previous experiments may have induced production of antibodies that recognise the native protein. The chemically denatured protein used could not have refolded inside or outside the adjuvant as both the cysteines were irreversibly carboxymethylated. Lack of enzyme activity and susceptibility to proteolysis showed also that the protein was denatured. The antibodies from this rabbit that bound the native protein were mapped to CTRs that formed two groups on the surface. All the CTRs in first group were located in the loop region D85 to V119 with highest backbone mobility and also including the dominant epitope in the animals immunised with  $\beta$ -lactamase in Freund's adjuvant. The other region included the sequence in  $\beta$ -lactamase with the second highest backbone mobility (see Figure 4.7). This region did also have relative high response of cross-reactive antibodies in serum from rabbit 1 as shown in this chapter and was one of the few regions with antibody response in the four mice immunised with  $\beta$ -lactamase in Freund's adjuvant.

## 6.4 Conclusion

The  $\beta$ -lactamase was denatured in Freund's adjuvant. The amount of native  $\beta$ -lactamase in Freund's adjuvant went through an exponential decay with a half-life of about 20 minutes and some of the  $\beta$ -lactamase had probably also been denatured in the emulsification process. The vast majority of the  $\beta$ -lactamase was denatured when injected into the animals with Freund's adjuvant. It was further shown that the serum from rabbit 1 had cross-reactive antibodies which may have linked the denaturation of the protein to the epitope distributions seen when using Freund's adjuvant. Mapping of the cross-reactive antibodies or cross-reactive plus native-specific antibodies from this rabbit gave similar

epitope distributions. It was further shown that displaying denatured protein to the immune system could give rise to antibodies recognising native protein. These antibodies were directed against two regions on the surface and included the loop region with highest signals in the animals immunised with the  $\beta$ -lactamase in Freund's adjuvant.



# **Chapter 7**

## **Discussion**

### **7.1 Introduction**

The immune systems' ability to efficiently fight pathogens is augmented by production of antibodies that specifically bind the foreign antigens. Revealing which parts of the antigen that are recognised by antibodies is important for understanding how the humoral immune system works. It may also further help us in designing vaccines that aid the immune system to efficiently fight the pathogens. The data collected by the novel mapping method presented in this thesis give a systematic picture of the relative epitope distribution on the three-dimensional protein surface. The results also demonstrate serious flaws in earlier epitope studies. The work presented here will hopefully aid to a more precise evaluation of the relationship between the intrinsic properties of the antigen, the nature of inoculation and the epitope distribution.

### **7.2 Main discussion**

The aim of this work was to find the relative distribution of epitopes on a protein antigen and to understand the principles behind the epitope distribution. An important property of the presented epitope data to have in mind during the analysis is that only antibodies able to bind the native protein, native-specific and cross-reactive antibodies, were detected in the mapping assay. This property separates these data from all epitope data

based on fragments of the protein antigens. As pointed out in the introduction, a large portion of anti-protein antibodies do not bind protein fragments [Lando et al., 1982b].

Two rabbits and four mice were immunised with the model antigen  $\beta$ -lactamase in Freund's adjuvant. All six animals had the highest antibody response against the same loop region of the antigen. This biased antibody distribution is not likely to be the result of a random selection of the antigen surface in all six animals. There was further no indication of artefacts in the mapping assay that had caused the observed epitope dominance. Intrinsic properties of the protein must therefore somehow have influenced the epitope distribution. One possibility is that the repertoires of antibody receptors on unchallenged B cells were biased against some surface properties of the antigen. But there were results that indicated that the immunisation method used modified the epitope distribution. It was regarded as important to clarify what influence Freund's adjuvant may have on the epitope distributions as the vast majority of previous epitope studies with model antigens have been based on immunisation with this adjuvant.

Two rabbits were immunised with native protein in PBS to see what the epitope distributions look like without the influence of the adjuvant. Comparison of the serum antibodies from these rabbits with the serum antibodies from the rabbits immunised with Freund's adjuvant imply that the adjuvant did have an impact on the antibody distribution even though few animals were analysed. The antibodies produced after injection of  $\beta$ -lactamase in PBS were more evenly distributed over the antigen surface than antibodies produced after injection of  $\beta$ -lactamase in Freund's adjuvant. The relatively even epitope distribution or lack of dominant epitopes did not support that the repertoires of antibodies on unchallenged B cells were biased against antigen surfaces with particular properties. The results further implied that data from epitope studies based on immunisation with Freund's adjuvant, which includes the vast majority of earlier epitope studies with model antigens, need to be reinterpreted.

The sera from the rabbits injected with  $\beta$ -lactamase in Freund's adjuvant had some hints to how the adjuvant may have influenced the epitope distribution. There are good reasons to believe that the  $\beta$ -lactamase initially was native. It was resistant to proteolysis. No cleavage products were seen in the presence of trypsin. The carboxymethylated protein was in contrast very sensitive to proteolysis and was easily cleaved into smaller products. The protein used in the immunisation also had the same enzyme activity as the C-terminal cysteine mutant 308C. The latter contained no denatured protein as verified with a denatured-specific monoclonal antibody. The rabbit sera from the rabbits injected with  $\beta$ -lactamase in Freund's adjuvant contained antibodies that bound strongly to denatured protein. Some of these antibodies were only able to bind the protein in denatured state. (The volumes of the mouse sera were too small for a detailed analysis.) This showed that denatured antigen had been presented to the immune system.

Results from two independent methods demonstrated that Freund's adjuvant denatured  $\beta$ -lactamase. The rate at which  $\beta$ -lactamase lost its enzyme activity in the adjuvant showed that the vast majority of this antigen was denatured before injected into the animals. A significant amount of the denatured antigen was also covalently cross-linked in the adjuvant. Although some of the denatured monomeric protein may have folded back to native conformation when released from the adjuvant it was very unlikely to be the case for the covalently linked antigen. The presence of serum antibodies specific for either denatured or native protein suggests that both native and denatured antigen were presented to the immune system of the rabbits.

The most obvious difference in the specificities of the antibodies from the two groups of rabbits was that the antibodies produced in response to antigen in PBS bound poorly to denatured protein in contrast to the antibodies produced in response to antigen in Freund's adjuvant. Some of the antigen delivered in PBS may have been denatured before being presented to the B cells. But the lack of serum antibodies that bound the denatured protein, especially in rabbit 4, implied that very little, if any, of the protein was denatured. Lando et al. [1982b] showed that 30-40% of anti-sperm whale myoglobin

(Mb) antibodies did not recognise peptide fragments of Mb. Their data were based on serum from animals immunised with Mb in Freund's adjuvant. The results in this thesis suggest that the fraction of antibodies only binding native protein can be significantly higher if only native protein is used for immunisation. Lando and Reichlin [1982a] further demonstrated that the sera included denatured-specific antibodies which supports the assumption that denatured protein were present in their immunisation in with Mb in Freund's adjuvant. The change in amount of antibodies that bind denatured protein was also shown by Scibienski [1973] in immunisation experiments with hen egg lysozyme (HEL). He showed that antibodies produced in response to HEL emulsified in Freund's adjuvant bound both native and denatured HEL, and in contrast, that injection of HEL in PBS gave antibodies that bound native but not denatured HEL.

These results do also have important implications for epitope data collected with fragments of antigens when Freund's adjuvant is used in the immunisation. Most of the antibodies that recognised the linear form of the  $\beta$ -lactamase, including the cross-reactive antibodies, were produced in response to the protein which was denatured by the adjuvant. Freund's adjuvant has therefore affected the majority of epitopes detected by fragments of the antigen.

Freund's adjuvant was clearly connected to the denaturation of the protein. It was important to establish if this significant impact on the protein structure of denaturation by Freund's adjuvant was connected to the adjuvant's influence on the epitope distribution on the native protein. Activation and later selection of B cells is depending on their ability to bind the antigen. B cells specific for both denatured and native antigen may have had a selective advantage over B cells specific for a single form of the antigen if both forms of the antigen were present. Somatic mutation may have altered the B-cell receptors preference for the form of antigen and the specificities of the antibodies later in the immune response may therefore not have directly mirrored the distribution of antigen form that initiated the antibody production.

The presentation of denatured protein to the immune system may have influenced the epitope distribution in at least two ways. The B cells may initially have been induced by native protein to produce antibodies. The B-cell clones bearing cross-reactive antibody receptors could at later stages have been boosted by denatured antigen. Another explanation could be that denatured protein induced production of antibodies that also were able to bind some parts of the native protein.

Both theories require that cross-reactive antibodies were present at least in some stages of the immune responses. The presence of cross-reactive antibodies in the rabbits after immunisation with Freund's adjuvant therefore supports the theory that denatured protein may have influenced the epitope distribution. The distribution of the cross-reactive antibodies in rabbit 1 was very similar to the distribution pattern seen for all the antibodies specific for the native protein (including both cross-reactive and native-specific antibodies). This therefore supports that the cross-reactive antibodies were an important part of the epitope distributions. This also strengthens the theory that the antigen which was denatured by the adjuvant was connected to the epitope distribution on the native protein.

If the first of the two theories above is correct, that denatured protein can induce production of antibodies that recognise the native protein, then it should be possible to immunise with only denatured protein and subsequently detect antibodies on native protein. Immunisation with chemically and irreversibly denatured protein did give rise to antibodies that bind native protein even though with low affinities. Antibodies from this rabbit gave high signal on the same surface region which was dominating the response in the animals immunised with  $\beta$ -lactamase in Freund's adjuvant. This supports the theory of a connection between the denatured antigen and epitope distribution on the native antigen, even though it does not prove that the antibodies dominating the epitope distributions in the animal immunised with unmodified  $\beta$ -lactamase in Freund's adjuvant were induced by denatured or native antigen.

The conclusion that denatured protein can induce antibodies that recognise native protein is different from the hypothesis from Richard Lerner's group who claim that even small peptides are likely to induce production of antibodies that recognise native protein [Wilson et al., 1984, Green et al., 1982]. They analysed anti-peptide antibodies ability to bind the full size protein which was dried onto plastic plates and washed with methanol. These conditions are likely to denature the antigen molecules. This is confirmed by their results as one of the epitopes they detected this way is not accessible on the native protein.

The knowledge about what the relative epitope distributions looked like with and without Freund's adjuvant opened the possibility to predict how the adjuvant affects the epitopes on the different parts of the antigen surface and to explain the observed epitope patterns. The best clue to how the adjuvant may have influenced the epitope distribution is probably the positive correlation between backbone mobility and epitope distribution as observed with sera from the rabbits immunised with antigen in Freund's adjuvant. This correlation was almost absent for the sera from rabbits immunised with antigen in PBS and is therefore most likely reflecting an effect of the adjuvant. The dominant epitope observed in the six animals immunised with antigen in Freund's adjuvant was in a long loop region. This may be an important connection between the denatured protein and the epitope distribution on the native protein. A possible explanation is that antibodies raised against denatured protein were most likely to bind the regions on the native protein with high mobility since these regions would have had a better chance of adopting into predefined binding surfaces (paratopes) on the antibodies. This is supported by the results that showed that immunisation with irreversibly denatured protein can give rise to antibodies that bind native protein. The antibodies that followed from this immunisation recognised several surface regions on the native protein that clustered together in the two patches with highest backbone mobility. One might also think that antibodies raised against linear forms of the protein would bind sequential epitopes. The most sequential epitopes on the protein surface are loops and protruding regions [Barlow et al., 1986] which in general are also more flexible than regions with secondary

structure [Novotny et al., 1986]. Linear components of the antigen surface were therefore probably important factors for the antibody response to antigen administered in Freund's adjuvant. This is also supported by the fact that the isolated cross-reactive antibodies from rabbit 1 bound predominantly in the same long loop region. Cross-reactive antibodies were at very low levels or absent in the two rabbits immunised with antigen in PBS.

Westhof and his colleagues [Westhof et al., 1984] reported a positive correlation between the backbone mobility and linear epitopes in myoglobin, lysozyme and tobacco mosaic virus protein. The epitope data in their study were collected from animals immunised with the antigens in Freund's adjuvant. The results presented in this thesis suggest that the correlation can be an artefact of using Freund's adjuvant.

Although no strong correlation was detected in this work between backbone flexibility and epitope distribution from responses to native protein there may well be intrinsic properties of proteins that to some extent could bias the epitope distribution. The epitope response may have been the product of as a relatively even distribution on the antigen surface due to a random selection of B cell clones superimposed by biasing factors as seen with backbone mobility in the presence of Freund's adjuvant. The presentation of the complete antigen surface to the immune system, by injecting the antigen in PBS, reduced the chance of backbone mobility to modify the epitope distribution and only a weak, if any, correlation was detected with the backbone mobility.

The epitope mapping results from sera of the rabbits immunised with antigen in PBS support the conclusion in reappraisal by Benjamin et al. [1984] that the whole protein surface is antigenic or more precisely; "The surface of a protein antigen consists of a complex array of overlapping potential antigenic determinants; in aggregate these approach a continuum." The conclusion is based on potential antigenic residues reported in several earlier epitope studies. The results presented in this thesis did not show that the epitopes form a continuum on the surface, but verified that most of the antigen sur-

face is antigenic. Benjamin and his colleagues may well be wrong in arguing that their discussion is limited to studies where antibodies were elicited by immunisation by native protein. At least the majority of the studies they discuss were based on immunisation with Freund's adjuvant, which is likely to denature several antigens as discussed below. But there are no indications in the data presented in this thesis which indicate that their main conclusion is wrong.

The collection of data presented in the reappraisal by Benjamin and his colleagues establishes which parts of the antigen surfaces are recognised by antibodies but it did not provide information on the relative importance of each epitope. The results presented in this work extend the knowledge on epitope distribution by adding information on the relative concentrations of antibodies to different parts of the antigen surface. The fairly even distribution observed in animals immunised with native protein suggests that the immune system has evolved to recognise all protein surfaces equally well. This may be an important aspect of the immune systems battle against pathogens, for example to avoid that pathogens escape antibody recognition through a selection of surface properties that are unfavourable for binding of the antibody repertoire.

An aspect of the relative epitope data that should be considered is the kind of distribution they represent. Assays based on ELISA measure combinations of the antibody concentrations and affinities. The antigen was in the ELISA format used in this work presented on a solid phase. This did probably allow bivalent binding of the IgG molecules. Most antibodies with affinities above a low threshold (not quantified) were expected to bind and contribute to the signal in such epitope mapping assays. This means that the epitope data presented here most likely represent the concentrations of antibodies to the different regions of the antigen surface. Data that represent the distribution of epitope affinities on the antigen surface may reveal more about the antigenicity distribution.

An important question to raise in this context: Are other protein antigens than  $\beta$ -lactamase denatured by Freund's adjuvant. Results from two independent methods



showed that  $\beta$ -lactamase was extensively denatured by Freund's adjuvant. This was also supported by specificities of the antibodies produced against  $\beta$ -lactamase in Freund's adjuvant or PBS. Proteolysis experiments showed that also bovine serum albumin (BSA), a commonly used model antigen, was denatured by Freund's adjuvant. BSA lost its proteolytic resistance to trypsin, in the same way as  $\beta$ -lactamase, when emulsified in the adjuvant. This was not shown for the commonly used antigens horse cytochrome c or hen egg lysozyme (HEL). The results from the immunisation studies carried out by Scibienski [1973] with HEL (described earlier) suggest that Freund's adjuvant denatures HEL. It can therefore not be excluded that other proteins, like cytochrome c, may be denatured by Freund's adjuvant without detectable loss in resistance to trypsin with the method used here. Immunisation with sperm whale myoglobin (Mb) in Freund's adjuvant carried out by Lando and his colleagues [Lando and Reichlin, 1982a] gave rise to a fraction of antibodies only binding the antigen in denatured form. Denatured protein must therefore have been presented to the immune system. They showed that antibodies isolated by fragments of the protein did not bind whole Mb which implies that the protein they also used for immunisation was native prior to the emulsification in Freund's adjuvant and further that the protein was denatured in the immunisation process. Not all proteins are necessarily denatured by Freund's adjuvant. The electromagnetic resonance spectrum of the heme group in hemoglobin is dependent on the folding state of the protein. Berzofsky et al. [1976] examined electromagnetic resonance spectrum of hemoglobin in Freund's adjuvant and found no denaturation of the protein.

The inoculation regimes may influence the epitope distributions. Delivering the model antigen in Freund's adjuvant did probably resemble a typical infection by the slow release of antigen due to the stability of the adjuvant in the animals [Herbert, 1968]. The antigen injected in PBS was, in contrast, instantly accessible to extracellular liquid and was probably displayed to the immune system in a shorter period. Injection of  $\beta$ -lactamase in PBS did still induce secondary antibody responses as judged by the titers of IgG [Bandilla et al., 1969]. Most immunisation methods with simple antigens will have artificial elements. Although injecting antigen in PBS is not the optimal for mod-

elling a natural infection, complication as denaturation or other epitope discriminating factors were avoided.

To study the relationship between intrinsic protein properties and antigenicity it is of great advantage to avoid host regulation due similarities between antigen and the host proteins. A lot of the previous collected epitope mapping data are based on model antigens with homologues in the immunised species as cytochrome c [Jemmerson and Margoliash, 1979], serum albumin [Benjamin and Teale, 1978], myoglobin [East et al., 1982] and lysozyme [Metzger et al., 1984]. The study by Jemmerson and Margoliash [1979] clearly demonstrated the influence of self-protein on the epitope distribution on a homologue antigen. They fractionated antibodies from rabbit serum against horse cytochrome c into seven subpopulations, comprising all the cytochrome c specific antibodies, by using other cytochrome c homologues. The binding of all seven sub-populations was dependent on four of the six amino acids where rabbit and horse cytochrome c differ. The two remaining positions are adjacent to the other four. The rabbits produced antibodies to the regions where the antigen differed from the self-protein (homologue). The antibodies produced in these situations will obviously be the outcome of a combination of initial generation of antibody specificities and host regulatory mechanisms. Information on epitope distributions, for example dominant epitopes, identified in such immune responses will only have meaning when described in relation to the host's homologue protein. Data from these studies are not useful for evaluating (or predict) relative epitope distribution based on the antigen structure only. The topographic epitopes presented in this thesis are mapped on a model antigen with no expected homologue in the immunised species as  $\beta$ -lactamase is bacterial protein.  $\beta$ -Lactamase was in this study treated as a completely foreign antigen which dramatically simplified the connection between observed epitopes and properties of the antigen surface.

### 7.3 Final conclusion

A method was developed to determine the antigenicity of different regions of the surface of the model antigen  $\beta$ -lactamase. The method involved the construction of a set of mutant antigens in which cysteine residues had been introduced at different points in the surface of the antigen. Each mutant protein was tethered through the surface cysteine residue and a bifunctional chemical cross-linker to solid phase, allowing the creation of an array of mutant antigens, each oriented so as to expose or to mask different topological regions of the antigen surface. The 23 mutants prepared represented a relatively even distribution of tethering points on the antigen surface, which at most should have about 13 mutually exclusive antibody epitopes. All the mutants had enzyme activity which demonstrated that they all contained native protein. Binding of a set of monoclonal antibodies to the mutants was further shown to be sensitive to the orientation of the antigen. Presentation of the array of oriented antigen to rabbit or mouse serum allowed binding and subtraction of serum antibodies that were not blocked by the cross-linker. The remaining antigen specific antibodies, representing the epitopes that were masked in each orientation, could then be quantified through binding to the antigen oriented to display these epitopes. The  $\beta$ -lactamase used to quantify the antibodies contained no denatured protein as verified by a monoclonal specific for the denatured form of the protein. The epitope data were therefore limited to the antibodies that bound native protein. The relative epitope data from this assay represented topological maps of the antibody responses and could be used to compare the response in different animals to the three-dimensional antigen surface.

When the immunisations were undertaken with use of Freund's adjuvant, the antibody response to  $\beta$ -lactamase was mainly directed against a region centered on a flexible loop. The loop region dominated the antibody response in all six animals immunised this way. The sera furthermore comprised antibodies cross-reactive to both native and denatured protein. These cross-reactive antibodies also dominantly bound the flexible loop. By contrast, in the absence of Freund's adjuvant, the antibody response was more evenly

distributed, and with few cross-reactive antibodies. This suggested that Freund's adjuvant denatures  $\beta$ -lactamase and that the appearance of dominant epitopes may follow the presentation of denatured protein to the immune system. Analysis of the enzyme activity of  $\beta$ -lactamase in Freund's adjuvant showed extensive denaturation of the protein in the adjuvant and a half-life of the native protein of about 21 minutes. The denaturation was also confirmed through the protein's loss of resistance to proteolysis when mixed with the adjuvant. The denatured antigen's ability to induce or boost antibody clones specific for flexible loops on the native protein was also supported by the data showing that a rabbit immunised with irreversibly denatured protein produced antibodies which recognised native protein. Mapping of the epitopes on the native  $\beta$ -lactamase showed that these antibodies bound the two parts of the protein with the highest backbone flexibility. This included the same loop region which dominated the antibody response in the six animals immunised with the antigen in Freund's adjuvant. The observations have implications for understanding the basis for dominance of B-cell epitopes, and also for design of vaccines.

# Chapter 8

## Materials and Methods

For a list of chemical and material see page 161.

### 8.1 General methods

#### 8.1.1 PCR amplification

50  $\mu$ l reactions were prepared containing 25 pmol of each primer, 2.5 units of superTaq DNA polymerase (Enzyme Technologies), 200  $\mu$ M of each dNTP and the recommended buffer (50 mM KCl, 10 mM Tris·HCl pH 8.8, 1.5 mM  $MgCl_2$ , and 0.1% Triton X-100) and DNA from a picked colony or purified DNA as templates. The samples were denatured for five minutes at 94°C followed by 30 cycles: 94°C for one minute, 55°C for one minute and 72°C for one minute. The cycles were followed by five minutes at 65°C. The lid was kept at 105°C.

#### 8.1.2 DNA sequencing analysis

The  $\beta$ -lactamase gene was amplified with 30 cycles of PCR synthesis using the primers 5'-CAG GAA ACA GCT ATG AC-3' and 5'-GTA AAA CGA CGG CCA GT-3'. The PCR products were purified with Qiagen QIAquick PCR purification kit. Sequencing was performed using the dideoxy chain termination method [Sanger et al., 1977] with ABI PRISM BigDye terminator mix. The Sequencing reactions were primed with the primers

5'-CAG GAA ACA GCT ATG AC-3', 5'-GTA AAA CGA CGG CCA GT-3', 5'-GAG CAA CTC GGT CGC CGC ATA-3' and 5'-TTC CCG GCA ACA ATT AAT AGA-3'. Sequences were analysed on an Applied Biosystems 373 Automated DNA sequencer (Perkin Elmer). DNA sequence analysis was conducted using SeqEd (Applied Biosystems).

### 8.1.3 Protein gels

Protein gels were Nowex NuPage 10% or 12% precast Bis-Tris polyacrylamide gels. The electrophoresis was done at 200 V for 35-40 minutes. The buffer was either MES (50 mM 2-N-morpholine ethane sulfonic acid, 50 mM Tris Base, 3.5 mM sodium dodecyl sulphate (SDS), 1 mM EDTA) or MOPS (50 mM 2-N-morpholine propane sulfonic acid, 50 mM Tris Base, 3.5 mM SDS, 1 mM EDTA). Antioxidant, supplied by the manufacturer, was used in the upper chamber at recommended concentration. Samples were reduced by 50 mM dithiothreitol (DTT). The gels were stained for one hour with Coomassie (0.1% Coomassie R-250 (from powder) dissolved in 45.5% methanol and 4.6% acetic acid) after incubation in 45.4% methanol and 4.6% acetic acid. The gels were destained in 5% methanol and 7.5% acetic acid over night.

### 8.1.4 Detection with antibodies conjugated to HRP

ELISA plates with antibodies conjugated to horseradish peroxidase (HRP) were washed three times with PBS containing 0.1% Tween (0.1%TPBS) and once with PBS before developing with 100  $\mu$ l TMB (100  $\mu$ g/ml 3,3',5,5'-tetramethylbenzidine in 100 mM sodium acetate pH 6.0 and 1:5000 (v/v) 30% hydrogen peroxide). Adding 50  $\mu$ l 1 M H<sub>2</sub>SO<sub>4</sub> stopped the peroxidase reaction. The colour change was measured by OD<sub>450nm</sub>-OD<sub>650nm</sub>.

## 8.2 Specific methods for chapter 2

### 8.2.1 Production of monoclonal antibodies

The production of monoclonal antibodies was carried out under close supervision and help at the Central Laboratory, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway.

The production of monoclonal antibodies were essentially done as described in [Harlow and Lane, 1988]. A Balb/C mouse was injected subcutaneously twice with 100  $\mu\text{g}$   $\beta$ -lactamase in Freund's complete adjuvant and once with 30  $\mu\text{g}$   $\beta$ -lactamase in Freund's incomplete adjuvant. (The production of  $\beta$ -lactamase is described in section 8.2.5.) Each injection was separated by three weeks. The cell fusion was done four weeks after the injection with Freund's incomplete adjuvant. The mouse was injected intravenously each of the four days prior to the cell fusion with 50  $\mu\text{g}$ , 200  $\mu\text{g}$ , 200  $\mu\text{g}$  and 200  $\mu\text{g}$   $\beta$ -lactamase in PBS respectively. These injections were done intravenously.  $2.4 \times 10^7$  NS0 cells were mixed with  $5 \times 10^7$  spleen cells, fused with PEG 3,000-3,700 and plated in 10 96-well plates with HAT (hypoxanthine, aminopterin and thymidine) selection medium. The cells were tested after 10 days for antibody production: Media from the cell cultures were analysed for the presence of antibodies that bound to native or denatured  $\beta$ -lactamase. The native protein was biotinylated and captured on streptavidin coated plates. The denatured protein was obtained by coating the  $\beta$ -lactamase directly on MaxiSorp 96-well plates in PBS and the plates were dried over night. (The absence of native protein was verified with the chromogenic substrate Nitrocefin and mouse serum was used as a positive control for the presence of  $\beta$ -lactamase on the plates.) Hybridoma cells were then selected for their ability to produce antibodies that gave high signal on native, denatured or both forms of the protein. The selected cells were sub-cloned and retested for expression of specific IgG antibodies. IgG antibodies were purified on IgG columns and stored with 0.02% sodium azide.

## 8.2.2 Construction of the expression vector for $\beta$ -lactamase

### pHEN-1btl

The  $\beta$ -lactamase gene, amp-C, was cloned from a vector derived from pBR322. The sequence was amplified by PCR with the primers 5'-CCC AAG CTT GGG ATG CTT CAA TAA TAT TG-3' and 5'-CCT CAC TGA TTA AGC ATT GGG CTC TAG ATT ACA AGG ATG ACG ATG ACA AGC ATC ATC ATC ACC ATC ACT AAG AAT TCC GG-3'. The product included the ribosomal binding site and leader sequence from pBR322. The gene product was followed by a FLAG tag, His (6) tag and an ochre stop codon (see figure B.1, page 158). The gene was cloned down stream of a lac promoter in the plasmid pUC119 between restriction sites HindIII and EcoRI. The ampicillin resistance gene in pUC119 was replaced by chloramphenicol resistance as in pBC-SK+ (Stratagene). (The AflIII-XmnI fragment of pBC-SK+ containing the cat gene and the ColE1 replication origin was ligated to the pUC AflIII-AatII fragment (AatII site trimmed blunt with T4 DNA polymerase)). The pUC119 based vector with the chloramphenicol resistance (pRooster) was a gift from Dr. Peter Wang. The cloned  $\beta$ -lactamase gene was checked by DNA sequence analysis as described in section 8.1.2.

## 8.2.3 Site specific mutations

The mutations were carried out after the principles described in [Kunkel, 1985].

### Preparation of ssDNA

The  $\beta$ -lactamase expression plasmid pHEN-1btl was electroporated into the bacterial strain BW313 (*dut*<sup>-</sup>, *ung*<sup>-</sup>, F') and plated on an agar plate with chloramphenicol. A single bacterial colony was used to inoculate 5 ml media (2xTy, 10  $\mu$ g/ml chloramphenicol and 1% glucose). This was incubated shaking over night at 37°C. 0.5 ml of the culture was used to inoculate 50 ml media (2xTy, 10  $\mu$ g/ml chloramphenicol and 1% glucose). This was incubated shaking until the culture reached an OD<sub>600nm</sub> of 0.4.  $3 \times 10^{11}$  KM13 [Kristensen and Winter, 1998] helper phage were added to the bacterial culture and incubated without shaking at 37°C for 30 minutes. The cells were spun



down and resuspended in 300 ml media (2xTy, 10  $\mu\text{g/ml}$  chloramphenicol, 50  $\mu\text{g/ml}$  kanamycin, 25  $\mu\text{g/ml}$  uridine, 1% glucose) and grown over night shaking at 30°C. The cells were spun down and the supernatant with phage particles were filtered with a 0.45  $\mu\text{m}$  filter. 75 ml PEG solution (20% polyethylene glycol and 2.5 M NaCl) was added to precipitate the phage particles. The pellet was resuspended in 10 ml TE buffer pH 7.0. 2.5 ml PEG solution was added to precipitate the phage particles. The pellet was then resuspended in 3 ml TE buffer pH 7.0. This resulted in  $1.7 \times 10^{12}$  phage particles per millilitre. ssDNA was then extracted by mixing the phage solution with equal volume of phenol followed by the same volume of chloroform.

### Site specific mutagenesis

A oligonucleotide containing the mutated codon plus typically 20 bases on each side and a 5'-end phosphorylation were mixed with the vector in SSC buffer in a volume of 20  $\mu\text{l}$ . (The primers used were: K34C: 5'-CAA CTG ATC TTC AGC ATC GCA TAC TTT CAC CAG CGT TTC, N52C: 5'-TCT CAA GGA TCT TAC CGC TGC AGA GAT CCA GTT CGA TGT AAC C, K55C: 5'-GCG AAA ACT CTC AAG GAT GCA ACC GCT GTT GAG ATC CAG, E63C: 5'-GCT CAT CAT TGG AAA ACG TTC ACA GGG GCG AAA ACT CTC AAG G, Q90C: 5'-GTG TAT GCG GCG ACC GAG GCA CTC TTG CCC GGC GTC AAT AC, N100C: 5'-GTG AGT ACT CAA CCA AGT CAC ACT GAG AAT AGT GTA TGC G, E110C: 5'-CCA TCC GTA AGA TGC TTA CAT GTG ACT GGT GAG TAC TC, T114C: 5'-TCT CTT ACT GTC ATG CCA TCA CAA AGA TGC TTT TCT GT GAC TG, T140C: 5'-CTT CGG TCC TCC GAT CGT ACA CAG AAG TAA GTT GGC CGC AG, K146C: 5'-GTG CAA AAA AGC GGT TAG CTC GCA CGG TCC TCC GAT CGT TGT CAG, N154C: 5'-GTT ACA TGA TCC CCC ATG CAG TGC AAA AAA GCG GTT AGC TC, N175C: 5'-GTG GTG TCA CGC TCG TCG CAT GGT ATG GCT TCA TTC AGC TC, E197C: 5'-GGG AAG CTA GAG TAA GTA GAC AGC CAG TTA ATA GTT TGC GC, L201C: 5'-GGG AAG CTA GAG TAA GTA GAC AGC CAG TTA ATA GTT TGC GC, A202C: 5'-CTA TTA ATT GTT GCC GGG AAC ATA GAG TAA GTA GTT CGC CAG, K215C: 5'-CAG AAG TGG TCC TGC AAC ACA ATC CGC CTC CAT CCA GTC, A227C: 5'-ATC AGC AAT AAA CCA GCC ACA CGG AAG GGC CGA GCG CAG, E240C: 5'-GAT ACC GCG AGA CCC ACG GCA ACC GGC TCC AGA TTT

ATC AG, D254C: 5'-GAT ACG GGA GGG CTT ACC ACA TGG CCC CAG TGC TGC AAT G, Q269C: 5'-GTT CAT CCA TAG TTG CAC AAC TCC CCG TCG TGT AG, T271C: 5'-GTC TAT TTC GTT CAT CCA TAC ATG CCT GAC TCC CCG TCG TG, E281C: 5'-GCT TAA TCA GTG AGG CAC CTA TAC AAG CGA TCT GTC TAT TTC GTT CAT CC and K288C: 5'-GTA ATC TAG AGC CCA ATG ACA AAT CAG TGA GGC ACC TAT CTC.) A sample tube with each primer was placed in a 500 ml beaker of water at 70°C. This was allowed to cool to room temperature. T4 DNA polymerase buffer (from supplier) was then added and the volume was adjusted to 100  $\mu$ l. The tube was placed on ice and 2.5 U T4 DNA polymerase and 2 U T4 DNA ligase were added. After five minutes on ice and five minutes at room temperature the sample was incubated at 37°C for two hours. 3  $\mu$ l 0.5 M EDTA was added to terminate the reaction. 10  $\mu$ l was then used to transfect 100  $\mu$ l of CaCl<sub>2</sub> competent E.coli HB2151. The mutation was then checked by DNA sequencing analysis as described in section 8.1.2.

## 8.2.4 Expression and biotinylation of cysteine mutants

Bacteria from a single colony of HB2151 with the  $\beta$ -lactamase expression plasmid pHEN-1btl was used to inoculate 10 ml media (2xTy, 10  $\mu$ g/ml chloramphenicol and 1% glucose). This was incubated shaking over night at 37°C. The 10 ml culture was used to inoculate 1000 ml media (2xTy, 10  $\mu$ g/ml chloramphenicol and 0.1% glucose). This was incubated shaking until the culture reached an OD<sub>600nm</sub> 0.9. IPTG was added up to 1 mM and the culture was incubated shaking for five hours at 30°C. The bacteria were pelleted by centrifugation and then resuspended in 50 ml 20% sucrose, 30 mM Tris·HCl pH 8.0 and 1 mM EDTA. This was incubated on ice for 20 minutes and the bacteria were pelleted by centrifugation. The supernatant was kept on ice and the bacteria were resuspended in 50 ml 5 mM MgSO<sub>4</sub>. This was incubated on ice for 20 minutes and the bacteria were pelleted by centrifugation. The supernatants from both rounds were mixed and dialysed over night in 50 mM phosphate buffer pH 7.5, 500 mM NaCl and 20 mM imidazole (IMAC buffer, 20 mM imidazole). DTT was added to a concentration of 1 mM. The supernatant was then filtered with 0.45  $\mu$ m filter and loaded onto a 1 ml

Nickel-NTA column prewashed with IMAC buffer (250 mM imidazole) and equilibrated with imidazole IMAC buffer (20 mM imidazole). The column was washed with 20 ml IMAC buffer (20 mM imidazole, 1 mM DTT) followed by 10 ml IMAC buffer (20 mM imidazole) and then 10 ml IMAC buffer (35 mM imidazole). The protein was eluted with IMAC buffer (100 mM imidazole) into tubes on ice containing EDTA to a final concentration of 1 mM EDTA. The protein concentration was determined by  $OD_{280nm}$ , absorption coefficient  $29\,400\text{ M}^{-1}\text{cm}^{-1}$  [Sigal et al., 1984]. 1.5 times excess of HPDP (4 mM in dimethyl sulfoxide) linker was added and incubated rotating for two hours at room temperature. The samples were loaded on PD10 desalting columns and eluted with PBS with 1 mM EDTA. The samples were dialysed into 1xPBS, 50% glycerol and 1 mM EDTA. The biotinylated protein was stored at  $-20^{\circ}\text{C}$ .

### 8.2.5 Expression of unbiotinylated $\beta$ -lactamase

The same procedure was used as described in section 8.2.3 until the protein was loaded onto the Nickel-NTA column. The column was washed with 10 ml IMAC buffer (20 mM imidazole) followed by 10 ml IMAC buffer (35 mM imidazole). The protein was eluted with IMAC buffer (100 mM imidazole). The protein concentration was determined by  $OD_{280}$ , absorption coefficient  $29\,400\text{ M}^{-1}\text{cm}^{-1}$  [Sigal et al., 1984]. The samples were dialysed into 1xPBS, 50% glycerol and 1 mM EDTA. The protein was stored at  $-20^{\circ}\text{C}$ .

### 8.2.6 State of $\beta$ -lactamase mutants

#### Analysed through enzyme activity

MaxiSorp 96-well plates were coated over night at  $4^{\circ}\text{C}$  with  $100\text{ }\mu\text{l}$   $1\text{ }\mu\text{g/ml}$  streptavidin in carbonate buffer pH 9.6. The plates were washed three times with PBS. The wells were then blocked with  $200\text{ }\mu\text{l}$  4% BSA in PBS (4%BPBS) for two hours at room temperature.  $100\text{ }\mu\text{l}$   $5\text{ }\mu\text{g/ml}$   $\beta$ -lactamase mutants in 0.1%BPBS was added and incubated shaking for two hours at room temperature. The plates were washed three times with PBS. The enzyme activity was assayed with  $100\text{ }\mu\text{l}$  Nitrocefin ( $50\text{ }\mu\text{g/ml}$  Nitrocefin in PBS). The light absorbency ( $OD_{490nm}-OD_{405nm}$ ) was measured every 30 seconds for five

minutes.

### Enzyme activity of wild type $\beta$ -lactamase

The enzyme activity was measured in PBS pH 7.4 at 22°C. Determination of  $K_m$  was carried out with  $5 \cdot 10^{-10}$  M  $\beta$ -lactamase and Nitrocefin concentrations between  $2.5 \cdot 10^{-4}$  M and  $2.5 \cdot 10^{-6}$  M. Three samples were measured for each concentration. The initial conversion rates for the different substrate concentrations were fitted to Michaelis-Menton equation.  $k_{cat}$  was determined with enzyme concentrations between  $1.5 \cdot 10^{-9}$  M and  $5 \cdot 10^{-10}$  M and a Nitrocefin concentration of  $1.25 \cdot 10^{-4}$  M. Wavelength 486 nm was used to detect the converted Nitrocefin. The extinction coefficient for Nitrocefin at 486 nm is  $20,500 \text{ M}^{-1}\text{cm}^{-1}$ . All rates were corrected by subtracting 14.8% of the rates detected at 390 nm, the absorption maximum for uncleaved Nitrocefin.

### Analysed with monoclonal antibodies

MaxiSorp 96-well plates were coated over night at 4°C with 100  $\mu\text{l}$  1 mg/ml streptavidin in carbonate buffer pH 9.6. The plates were washed three times with PBS. The wells were then blocked with 200  $\mu\text{l}$  4%BPBS for two hours at room temperature. 100  $\mu\text{l}$  5  $\mu\text{g/ml}$   $\beta$ -lactamase mutants in 0.1%BPBS was added and incubated shaking for two hours at room temperature. The plates were washed three times with PBS. 100  $\mu\text{l}$  of the monoclonal antibodies P64.1 and P65.2 diluted in 0.1%TPBS were added to the wells. The concentrations were 100 ng/well and 0.5  $\mu\text{l/well}$  (cell culture media) respectively. 100  $\mu\text{l}$  horseradish peroxidase conjugated anti-mouse antibodies diluted 1:2500 in 0.1%TPBS was added and incubated shaking for one hour. The horseradish peroxidase was detected as described in section 8.1.4.

### 8.2.7 Mapping the binding regions of monoclonal antibodies

MaxiSorp 96-well plates were coated over night at 4°C with 100  $\mu\text{l}$  1 mg/ml streptavidin in carbonate buffer pH 9.6. The plates were washed three times with PBS. The wells were then blocked with 200  $\mu\text{l}$  4%BPBS for two hours at room temperature. 100  $\mu\text{l}$  5

$\mu\text{g/ml}$  biotinylated cysteine mutants of  $\beta$ -lactamase in 0.1%BPBS was added and incubated shaking for two hours at room temperature. The plates were washed three times with PBS. Added monoclonal antibodies to the plate at concentrations that just saturated the antigen (predicted from separate dilution series). The bound monoclonal antibodies were detected with 1:5000 dilution of HRP conjugated goat anti-mouse antibodies in PBS as described in 8.1.4.

### **8.2.8 Capturing $\beta$ -lactamase from solution with monoclonal antibodies**

The assay was carried out in the same way as with serum antibodies (8.5.11) with the exceptions: Anti-mouse antibodies were used in stead of anti-rabbit and the antibodies were not affinity purified. The amount of monoclonal antibodies added was 2  $\mu\text{g}$  in 100  $\mu\text{l}$  0.1%TPBS.

## **8.3 Specific methods for chapter 3**

### **8.3.1 Immunisation and test-bleeds of rabbits**

The rabbits were all Murex LOP (English Full Lop crossed with New Zealand rabbits. The resulting hybrid has been crossed for several generations.) The rabbits were between 4 and 4.5 kg. All injections into the rabbits were done with 0.5 mg  $\beta$ -lactamase (expressed and purified as described in section 8.2.5). The barstar injected into rabbit 4 was a gift from Dr Chris Johnson. The samples were injected subcutaneously in four points on the back of the rabbits. Injections with antigen Freund's adjuvant were prepared as described in section 8.5.3. Immunisations with antigen in Freund's adjuvant: Freund's complete adjuvant was used in the first injection and Freund's incomplete adjuvant was used in subsequent injections. Immunisation with antigen in PBS: Injections with antigen in PBS were prepared by dialysing the  $\beta$ -lactamase into PBS and adjusting the concentration to 1 mg/ml. Test-bleeds were done 10 days after injection. Sodium azide was added to the sera to a concentration of 0.02%. The sera were stored at  $-20^{\circ}\text{C}$ .

Test-bleeds (pre-bleeds) taken prior to the first injection showed no antibodies specific for  $\beta$ -lactamase in ELISA.

### 8.3.2 Mapping method for conformational epitopes (beads)

#### Plates:

Mapping of epitopes in sera from rabbits 1, 3 and 4 and mice 1, 2, 3, and 4 were carried out with this method. For the other sera see below. MaxiSorp 96-well plates were coated over night at 4°C with 100  $\mu$ l 1 mg/ml streptavidin in carbonate buffer pH 9.6. The plates were washed three times with PBS. The wells were then blocked with 200  $\mu$ l 4%BPBS for two hours at room temperature. 100  $\mu$ l of 5  $\mu$ g/ml  $\beta$ -lactamase 308C (biotinylated through a C-terminal cysteine) or other biotinylated cysteine mutants of  $\beta$ -lactamase in PBS with 0.1%BPBS was added and incubated shaking for two hours at room temperature. The plates were washed three times with PBS.

#### Beads:

Per serum sample to deplete; mixed 20  $\mu$ l prewashed magnetic streptavidin beads with 10  $\mu$ g of biotinylated cysteine mutants of  $\beta$ -lactamase. Incubated shaking for one hour. The beads were then washed six times with 100  $\mu$ l 0.1%TPBS.

#### Depletion of specific serum antibodies:

Each serum sample plus the control were treated in the same well on a polyvinyl chloride plate (Falcon 3912). The sera were used at dilutions below saturation of antigen on ELISA plates. 120  $\mu$ l diluted serum (1:5000 to 1:30,000) in 0.1%TPBS was mixed with 20  $\mu$ l beads loaded with a biotinylated  $\beta$ -lactamase mutant and incubated for five minutes. The liquid was then separated from the beads. The beads were further washed twice with 0.1%TPBS for five minutes. The beads were then remixed with the serum sample. This was repeated five times. After the beads were removed a new 20  $\mu$ l batch of beads loaded with the same biotinylated  $\beta$ -lactamase mutant was then added and the same process were repeated. The same depletion procedure was carried out for all the

$\beta$ -lactamase cysteine mutants with separate serum aliquots.

#### **Detection of remaining serum antibodies:**

Each sample was diluted 1:2 in 0.1%TPBS and split in two (The serum concentrations stated in the figure legends are after dilution). 100  $\mu$ l was added to each well with  $\beta$ -lactamase biotinylated through a C-terminal cysteine or other cysteines in specific surface positions. The plates were incubated shaking for one hour and then washed three times with 0.1%PBS. 100  $\mu$ l 1:5000 horse radish peroxidase conjugated anti-rabbit IgG antibodies in PBS was added and incubated for one hour shaking. The horseradish peroxidase was detected PBS as described in section 8.1.4.

### **8.3.3 Mapping method for conformational epitopes (plates)**

This method was used for sera from rabbit 2 and rabbit 5. 100  $\mu$ l 1  $\mu$ g/ml  $\beta$ -lactamase 308C (biotinylated through a C-terminal cysteine) or other biotinylated cysteine mutants of  $\beta$ -lactamase in 0.1%BPBS was added to precoated streptavidin plates (High Bind, Roche) and incubated shaking for two hours at room temperature. The plates were washed three times with PBS. A sample of 120  $\mu$ l rabbit serum diluted (1:12,500) 0.1%TPBS was added to a well with a biotinylated  $\beta$ -lactamase mutant and incubated shaking for 15 minutes. The sample was transferred to another well with the same biotinylated  $\beta$ -lactamase mutant. This was repeated for 11 wells. The sample was then transferred to a last well with the biotinylation mutant used for detection (308C, biotinylated through a C-terminal cysteine) and incubated shaking for one hour. The same procedure was carried out for all the  $\beta$ -lactamase cysteine mutants with separate serum aliquots. The plates were washed three times with 0.1%PBS. 100  $\mu$ l 1:5000 horse radish peroxidase conjugated anti-rabbit IgG antibodies in PBS was added and incubated for one hour shaking. The horseradish peroxidase was detected PBS as described in section 8.1.4.

### 8.3.4 Detecting epitopes by enzyme activity of $\beta$ -lactamase

Serum antibodies were specifically depleted in 100  $\mu$ l serum aliquots for all the 23 mutants as described in section 8.3.2. 10  $\mu$ l beads with  $\beta$ -lactamase 308C (see section 8.3.2) was added to each aliquot and incubated shaking for 20 minutes. The beads were then separated from the liquid with a magnet and washed once with 0.1%TPBS. The antibodies were eluted by resuspending the beads in ice cold 35  $\mu$ l glycine buffer pH 2.5 with 0.1% Tween and incubated for 30 seconds. The buffer was separated from the beads and mixed with 65  $\mu$ l ice cold 1 M Tris-HCl buffer pH 7.5. The eluted antibodies were captured on an ELISA plate pre-coated with goat anti-rabbit (100  $\mu$ l at concentration 10  $\mu$ g/ml in PBS over night and blocked with 200  $\mu$ l 4%BPBS for two hours). The plate was incubated for one hour shaking at room temperature followed by three washes with 0.1%TPBS. 100  $\mu$ l 1.5  $\mu$ g/ml  $\beta$ -lactamase 0.1%BPBS was added to the plates and incubated for one hour shaking at room temperature. The plate was washed three times in 0.1%TPBS. 100  $\mu$ l 50  $\mu$ g/ml Nitrocefin in PBS was added to each well. The light absorbency ( $OD_{490nm} - OD_{405nm}$ ) was measured every 30 seconds for five minutes.

### 8.3.5 Resolution of mapping assay

#### Specificity of antibody depletion

A MaxiSorp 96-well plates was coated over night at 4°C with 100  $\mu$ l 1 mg/ml streptavidin in carbonate buffer pH 9.6. The plate was washed three times with PBS. The wells were then blocked with 200  $\mu$ l 4%BPBS for two hours at room temperature. 100  $\mu$ l 5  $\mu$ g/ml  $\beta$ -lactamase biotinylated cysteine mutants of  $\beta$ -lactamase in 0.1%BPBS was added (one mutant in each well) and incubated shaking for two hours at room temperature. The plates were washed three times with PBS.

1 ml serum aliquots (1:2000 in 0.1%TPBS) were depleted for  $\beta$ -lactamase specific antibodies with L201C or A227C as described in section 8.3.2. The serum was diluted 1:3 in 0.1%TPBS. 100  $\mu$ l aliquots were added to 23 wells containing each of the biotinylated  $\beta$ -lactamase cysteine mutants. The plates were incubated shaking for one hour



and then washed three times with 0.1%PBS. 100  $\mu$ l 1:5000 horse radish peroxidase conjugated anti-rabbit IgG antibodies in PBS was added and incubated for one hour shaking. The horseradish peroxidase was detected PBS as described in section 8.1.4.

### 8.3.6 Protein fragmentation (for western blots)

#### Partial digest by CNBr

3.95 mg  $\beta$ -lactamase was dialysed into Millipore purified water. The final protein concentration was 5.5 mg/ml. Formic acid was added to a final concentration of 70%. The sample was split in two. 80  $\mu$ l of 60 mg/ml cyanogen bromide (CNBr) in acetonitrile was added to the protein solution. The sample was incubated for one hour. 1.6 ml Millipore purified water was added to the samples. The samples were dried in a centrifuge coupled to a vacuum pump. 400  $\mu$ l Millipore purified water was added to the samples. The samples were again dried in a centrifuge coupled to a vacuum pump. The fragmented protein was dissolved in 5% SDS and diluted to 0.5% SDS with Millipore purified water. The sample was stored frozen until use.

#### Partial digest by trypsin

2.25 mg  $\beta$ -lactamase was dialysed into 100 mM Tris-HCl pH 8.5 and 1 mM EDTA. The final protein concentration was 8.7 mg/ml. 94.2  $\mu$ l 8 M urea and 22.5  $\mu$ l of 1 mg/ml trypsin in 1 mM HCl was added to the  $\beta$ -lactamase. This gave a urea concentration of 2 M and a trypsin to  $\beta$ -lactamase ration of 1:100. The sample was incubated for 90 minutes at room temperature. The proteolytic activity of trypsin was stopped by adding PEFA-block up to a final concentration of 3.3 mM. The sample was stored frozen until use.

#### Partial digest by Lys-C

620  $\mu$ g  $\beta$ -lactamase was dialysed into 25 mM Tris-HCl pH 8.5 and 1 mM EDTA. The final protein concentration was 4.8 mg/ml. SDS was added to a final concentration of 0.5% and the sample was heated to 70°C for 10 minutes. The sample was cooled to room

temperature. 10  $\mu$ l of 0.5 mg/ml Lys-C was added and the sample was incubated for six hours at 37°C. The sample was stored frozen until use.

### 8.3.7 Western-blots with rabbit sera

Partial digest of  $\beta$ -lactamase (1  $\mu$ l cyanogen bromide digest, 0.6  $\mu$ l Lys-C digest or 4  $\mu$ l trypsin digest) were run on 12% Bis-Tris gel with MES buffer for 40 minutes at 200 V (see section 8.1.3). The protein was transferred to nitro-cellulose membranes under an electric field of 30 volts for one hour. The membranes were blocked over night at room temperature with 6% Marvel and 4%BPBS. The membranes were washed three times in PBS. Serum was added in 3% Marvel in 2%BPBS and incubated for one hour shaking at room temperature. The membranes were washed three times in PBS. Anti-rabbit IgG antibodies were added in 3% Marvel in 2%BPBS and incubated for one hour shaking at room temperature. The membranes were washed three times in PBS each with five minutes incubation. The membranes were developed by using ECL and X-Omat Film Kodak.

## 8.4 Specific methods for chapters 4 and 5

Rabbit sera was obtained as described in section 8.3.1.

### 8.4.1 Endotoxin (LPS) test

BioWhittaker, Europe, carried out the analysis of LPS content in protein samples. The protein was dialysed into PBS with the same concentration and under the conditions as used for preparing the antigen for immunisation. The sample was then sent to BioWhittaker in a glass vial. The Kinetic Chromogenic Test (KQCL, test code 95-103) was carried out at dilution s 1:10, 1:100 and 1:1000. The result was read from dilution 1:1000.

### 8.4.2 Immunisation and test-bleeds of mice

All the mice were female BALB/c and at least six weeks when given the first injection. All injections were done with  $\beta$ -lactamase in PBS emulsified in Freund's adjuvant, as described in section 8.5.3. Freund's complete adjuvant was used in the first injection and Freund's incomplete adjuvant was used in subsequent injections. The injections were given with 21 weeks intervals. 100  $\mu$ g, 30  $\mu$ g and 20  $\mu$ g was given in the first, second and third injection respectively. A total volume of 200  $\mu$ l was injected subcutaneously into four points. Test-bleeds were taken seven days after injection. The sera were stored at -20°C. (Test-bleeds (pre-bleeds) taken prior to the first injection showed no antibodies specific for  $\beta$ -lactamase in ELISA.)

### 8.4.3 Correlation calculations: surface properties and antigenicity

#### Surface amino acids in a radius of 10Å

The surface amino acids were identified by the Lee and Richards [1971] model with a solvent probe of 1.4 Å. The surface amino acids were defined as residues with solvent accessibility above zero. These calculations were done with the CCP4 supported software AREAIMOL (<http://www.ccp4.ac.uk>). Amino acids within a 10 Å radius were identified with the software GRASP [Nicholls et al., 1991].

#### Backbone mobility

The B-values from the crystal structure of  $\beta$ -lactamase were used as data for the mobility of the protein atoms. The B-values for  $\beta$ -lactamase were obtained from the file 1btl.pdb downloaded from SCOP database (<http://scop.mrc-lmb.cam.ac.uk/scop/>). The average B-values for the backbone atoms of each amino acid were calculated with the CCP4 supported software BAVERAGE (<http://www.ccp4.ac.uk>). Contact residues between different  $\beta$ -lactamase molecules in the crystal structure were removed from the data set to eliminate false mobility data. The contact residues were identified by using the CCP4 supported software CONTACT (<http://www.ccp4.ac.uk>). Contact residues were defined as amino acids containing atoms with less than 3.6Å distance (hydrogen

atoms not included).

### **Hydrophilicity:**

Hydrophilicity values for the amino acids were obtained from Hopp and Woods [1981] and Kyte and Doolittle [1982].

### **Accessibility:**

The accessibility values were calculated with the CCP4 supported software AREAIMOL (<http://www.ccp4.ac.uk>) using a probe of 10 Å radius.

### **Correlation coefficients:**

The correlation coefficients were calculated using the software EXCEL (Microsoft).

## **8.5 Specific methods for chapter 6**

### **8.5.1 Denaturation of $\beta$ -lactamase on streptavidin plates**

MaxiSorp 96-well plates were coated over night at 4°C with 100  $\mu$ l 1 mg/ml streptavidin in carbonate buffer pH 9.6. The plates were washed three times with PBS. The wells were then blocked with 200  $\mu$ l 4%BPBS for two hours at room temperature. 100  $\mu$ l 5  $\mu$ g/ml  $\beta$ -lactamase 308C (biotinylated with a maleimide-biotin linker through a C-terminal cysteine) in 0.1%BPBS was added and incubated shaking for two hours at room temperature. The plates were washed three times with PBS. 120  $\mu$ l 0.1 M Tris·HCl pH 8.0, 10 mM DTT, 1 mM EDTA and 1 M guanidine hydrochloride was added, incubated for one hour at 37°C. The solution was then replaced with 120  $\mu$ l 0.1 M Tris·HCl, 10 mM iodoacetamide, 1 mM EDTA and guanidine hydrochloride pH 8.0, incubated for 30 minutes at room temperature. The plates were washed three times with PBS. The absence of native  $\beta$ -lactamase was tested with Nitrocefin (100  $\mu$ l 50  $\mu$ g/ml Nitrocefin in PBS, OD<sub>490nm</sub>-OD<sub>405nm</sub> measured). The presence of  $\beta$ -lactamase was detected with through the C-terminal FLAG tag and the M2 anti FLAG antibody conjugated to

horseradish peroxidase. The M2 gave 10% lower signal on denatured  $\beta$ -lactamase than the native  $\beta$ -lactamase on the plate.

### 8.5.2 ELISA with serum on native or denatured $\beta$ -lactamase

MaxiSorp 96-well plates were coated over night at 4°C with 100  $\mu$ l 1 mg/ml streptavidin in carbonate buffer pH 9.6. The plates were washed three times with PBS. The wells were then blocked with 200  $\mu$ l 4%BPBS for two hours at room temperature. 100  $\mu$ l 5  $\mu$ g/ml  $\beta$ -lactamase 308C (biotinylated with a maleimide linker through a C-terminal cysteine) 0.1%BPBS was added and incubated shaking for two hours at room temperature. The plates were washed three times with PBS. The protein was denatured as described in section 8.5.1. The absence of native protein in the wells with denatured protein was confirmed by monoclonal antibodies specific for the denatured form (as described in section 8.2.6) and by enzyme activity of  $\beta$ -lactamase. Sera was diluted in 0.1%BPBS. The bound IgG were detected as described in section 8.3.2.

### 8.5.3 Mixing antigen with Freund's adjuvant

#### Classic method

The  $\beta$ -lactamase was dialysed into PBS and the concentration adjusted to 1 mg/ml. Equal volumes of  $\beta$ -lactamase solution and Freund's adjuvant were added to a 2 ml glass syringe. The syringe was connected through a 90° sanitary adapter to a second glass syringe of the same size. The contents was pushed between the two syringes as fast as possible for three minutes. The integrity of the resulting emulsion was tested by dripping the emulsion into a glass beaker with water (described in Appendix 3 Weir, D. M., Ed., 1978). The emulsion remained as a compact droplet in the water.

#### Other methods: Magnetic spinner

125  $\mu$ l 1 mg/ml  $\beta$ -lactamase in PBS and 125  $\mu$ l Freund's incomplete adjuvant was added to a cryovial (1.8 ml) on ice. A small magnetic spinner in the solution was turned by a magnetic stirrer at 1200 rpm for three minutes.

**Other methods: Homogeniser**

250  $\mu$ l 1 mg/ml  $\beta$ -lactamase in PBS and 250  $\mu$ l Freund's incomplete adjuvant was added to a cryotube (1.8 ml). A homogeniser with a 5 mm probe was applied to the solution at 11,000 rpm for five minutes.

**8.5.4 Extracting  $\beta$ -lactamase from Freund's adjuvant**

Ether quickly breaks down the emulsion and separates the water phase from the oil phase. Hexane does also brake the emulsion but less efficient. More hexane needs to be added to break the same volume of emulsion. Adding mineral oil to the adjuvant dilutes the surfactant and therefore destabilises the emulsion. The water phase can then be separated from the emulsion by centrifugation.

**Extracting  $\beta$ -lactamase from the adjuvant with ether**

1 ml water-saturated ether was added to 50  $\mu$ l emulsion. The sample was vortexed for 20 seconds and centrifuged for 30 seconds at 16,100g. The bulk ether was aspirated off. The tube was left open to let the remaining ether evaporate.

**Extracting  $\beta$ -lactamase from the adjuvant with hexane**

1 ml hexane was added to 50  $\mu$ l emulsion. The sample was vortexed for 30 seconds and centrifuged for 30 seconds at 16,100g. The hexane was aspirated off. This was repeated until all the emulsion had broken down.

**Extracting  $\beta$ -lactamase from the adjuvant with mineral oil**

1 ml mineral oil was added to 50  $\mu$ l emulsion. The sample was vortexed for 30 second and centrifuged for 30 seconds at 16,100g. The oil was aspirated off. This was repeated until all the emulsion had broken down.

### 8.5.5 Assaying adjuvant induced denaturation by enzyme activity

The 600  $\mu\text{l}$  1 mg/ml  $\beta$ -lactamase in PBS was mixed with 600  $\mu\text{l}$  Freund's incomplete adjuvant as described in section 8.5.3. The emulsion was incubated at room temperature. The water phase and oil phase were then separated with ether as described in section 8.5.4. The enzyme activity in the water phase was assayed with the chromogenic substrate Nitrocefin. The light absorption at wavelength 490 nm was subtracted from the absorption at 405 nm. 50  $\mu\text{l}$  50  $\mu\text{g/ml}$  Nitrocefin in PBS was mixed with 50  $\mu\text{l}$  sample. The enzyme activity was determined by using a dilution series of  $\beta$ -lactamase with known concentration. The samples of interest were diluted to get enzyme activity corresponding to 0.25 to 1 ng native  $\beta$ -lactamase.

#### Impact of ether on $\beta$ -lactamase

50  $\mu\text{l}$  1 mg/ml  $\beta$ -lactamase and 900  $\mu\text{l}$  water-saturated ether was vortexed for 45 seconds. The enzyme activity was assayed by with a dilution series of the water phase against a dilution series of  $\beta$ -lactamase that had not been in contact with ether.

#### Testing for adjuvant substances that inhibits the enzyme activity

500  $\mu\text{l}$  PBS was emulsified with Freund's incomplete adjuvant and extracted by ether as described in section 8.5.3.  $\beta$ -lactamase in PBS was mixed with an equal volume of extracted PBS. The enzyme activity was in a dilution series of the protein was compared to the enzyme activity of a dilution series of  $\beta$ -lactamase without extracted buffer present.

### 8.5.6 Assaying adjuvant induced denaturation by proteolysis

#### $\beta$ -Lactamase assayed with trypsin

1 mg/ml in PBS  $\beta$ -lactamase was mixed with 1:100 (w/w) trypsin. 600  $\mu\text{l}$  of the  $\beta$ -lactamase/trypsin solution was mixed with 600  $\mu\text{l}$  Freund's incomplete adjuvant as described in section 8.5.3. The emulsion was incubated at room temperature. The water phase and oil phase were then separated with ether as described in section 8.5.3. 1  $\mu\text{l}$  100 mM PEFA-block was added to 20  $\mu\text{l}$  of extracted sample to stop the proteolytic activity

of the trypsin. 15  $\mu$ l of the sample was then mixed with 5  $\mu$ l 4xLDS sample buffer and 1  $\mu$ l 1 M DTT. This was incubated at 90°C for 10 minutes before loading on 12% Bis-Tris gels.

#### **$\beta$ -Lactamase assayed with modified trypsin**

The experiment was done as above but with trypsin cross-linked by hydrophilic polymers instead of standard trypsin. The ratio of modified trypsin to  $\beta$ -lactamase was 1:10 (w/w).

#### **$\beta$ -lactamase assayed with modified thermolysin**

The experiment was done as with trypsin but with thermolysin in presence of 2 mM  $\text{CaCl}_2$ . The ratio of modified thermolysin to  $\beta$ -lactamase was 1:50 (w/w). The thermolysin was inactivated by 1 mM phenanthroline.

#### **BSA, HEL and cytochrome c assayed with trypsin**

The experiments were done as with  $\beta$ -lactamase. The bovine serum albumin (BSA), hen egg lysozyme (HEL) and horse heart cytochrome c was obtained from Sigma. The ratio of trypsin to BSA was 1:100 (w/w). The ratio of trypsin to HEL or cytochrome c was 1:10 (w/w).

### **8.5.7 Detection of cross-reactive antibodies**

#### **Beads:**

Pre-washed magnetic streptavidin beads were mixed with 308C (1  $\mu$ g  $\beta$ -lactamase, biotinylated with a maleimide linker through a C-terminal cysteine, per 1  $\mu$ l beads). Incubated shaking for one hour. The beads were then washed six times with 100  $\mu$ l 0.1%TPBS. Some of the beads were then mixed with 1 M guanidine hydrochloride, 10 mM DTT, 1 mM EDTA and 0.1 M Tris-HCl pH 8.0 (100  $\mu$ l per 10  $\mu$ l beads) and incubated for one hour at 37°C. The liquid was then replaced with the same volume of 1 M guanidine hydrochloride, 10 mM iodoacetic acid, 1 mM EDTA and 0.1 M Tris-HCl pH 8.0 and incubated for 30 minutes at room temperature.



**Plates:**

Plates were prepared with native and denatured  $\beta$ -lactamase as described in section 8.5.1. 100  $\mu$ l diluted serum (1:500 to 1:20,000) in 0.1%TPBS was mixed with 20  $\mu$ l beads loaded with native or denatured  $\beta$ -lactamase and incubated for 15 minutes. The beads were then separated from the liquid and washed once with 0.1%TPBS. The beads were resuspended in ice cold 35  $\mu$ l glycine buffer pH 2.5 with 0.1% Tween and incubated 30 seconds. The buffer was separated from the beads and mixed with 65  $\mu$ l ice cold 1 M Tris-HCl buffer pH 7.5. The samples were transferred to wells containing native or denatured protein and incubated shaking for one hour. The plates were washed three times with 0.1%TPBS. 100  $\mu$ l 1:5000 horse radish peroxidase conjugated anti-rabbit IgG antibodies in PBS was added and incubated for one hour shaking. The plates were washed three times with 0.1%TPBS and once with PBS before developing with 100  $\mu$ l TMB (100  $\mu$ g/ml 3,3',5,5'-tetramethylbenzidine in 100 mM sodium acetate pH 6.0 and 1:5000 (v/v) 30% hydrogen peroxide). Adding 50  $\mu$ l 1 M H<sub>2</sub>SO<sub>4</sub> stopped the peroxidase reaction. The colour change was measured by OD<sub>450nm</sub>-OD<sub>600nm</sub>.

**8.5.8 Mapping of cross-reactive antibodies**

Serum antibodies from rabbit 1 were isolated against different surface regions of  $\beta$ -lactamase as described in 8.3.2. The native-specific antibodies were then affinity purified on native antigen as described in 8.3.4 with biotinylated  $\beta$ -lactamase 308C on streptavidin beads. 2 volumes (67  $\mu$ l) of the eluted antibodies aliquots were mixed with 1 volume (33  $\mu$ l) of 0.1%TPBS with 0.3% BSA. The aliquots were then transferred to an ELISA plate with denatured  $\beta$ -lactamase. The two forms of denatured  $\beta$ -lactamase used was carboxymethylated as described in 8.5.1 and dried  $\beta$ -lactamase as described in 8.2.1. (The absence of native protein was verified with the chromogenic substrate Nitrocefin.) The plates were incubated four one hour shaking at room temperature. The plates were washed three times with 0.1%PBS. 100  $\mu$ l 1:5000 horse radish peroxidase conjugated anti-rabbit IgG antibodies in PBS was added and incubated for one hour shaking. The horseradish peroxidase was detected PBS as described in section 8.1.4.

### 8.5.9 Denaturation of $\beta$ -lactamase for immunisation

The  $\beta$ -lactamase was prepared as described in section 8.2.5. The protein was then dialysed into 0.1 M Tris·HCl pH 8.0 over night. Added the following chemicals to the given concentrations; guanidine hydrochloride 3 M, 0.1 M Tris·HCl pH 8.0, EDTA 1 mM and DTT 10 mM. Incubated for four hours at 37°C. To one volume of protein sample; added four volumes of 3 M guanidine hydrochloride, 50 mM iodoacetic acid, 0.1 M Tris·HCl pH 8.0 and 1 mM EDTA and incubated for one hour at room temperature. The protein was then loaded on a Nickel-NTA column with 0.5 ml packed beads pre-equilibrated with 3 M guanidine hydrochloride and 0.1 M Tris·HCl pH 8.0. A gradient with 0.1 M Tris·HCl pH 8.0 and from 3 - 0 M guanidine hydrochloride was then run through the column. The column was finally washed with PBS and the protein eluted with PBS containing 250 mM imidazole. The protein was dialysed against PBS for two hours followed by dialysis against 50% glycerol, 1xPBS and 1 mM EDTA over night at 4°C. The sample was stored at -20°C. The denatured protein was dialysed against PBS over night before used for immunisation. The protein concentration was determined by OD<sub>280</sub>, assuming the same absorption coefficient 29 400 M<sup>-1</sup>cm<sup>-1</sup> as the native protein. The amount of protein was also estimated on a SDS gel.

### 8.5.10 Circular dichroism spectroscopy

The protein was in PBS. The protein concentration was 0.60 mg/ml. 0.5 mm cuvettes were used. 10 scans were executed for each sample. The heat denatured protein was at 90°C. All other samples were at 20°C. The samples were analysed with a Jasco J-720 Spectropolarimeter.

### 8.5.11 Capturing $\beta$ -lactamase from solution with serum antibodies

MaxiSorp 96-well plates were coated over night at 4°C with 100  $\mu$ l 1 mg/ml goat anti-rabbit antibodies. The plates were washed three times with PBS. Affinity purification of  $\beta$ -lactamase specific antibodies: 100  $\mu$ l diluted serum 1:200 in 0.1%TPBS was mixed with 20  $\mu$ l beads loaded with native  $\beta$ -lactamase (as described in section 8.3.2) and

incubated for 15 minutes. The beads were then separated from the liquid and washed once with 0.1%TPBS. The beads were resuspended in ice cold 35  $\mu$ l glycine buffer pH 2.5 with 0.1% Tween and incubated 30 seconds. The buffer was separated from the beads and mixed with 65  $\mu$ l ice cold 1 M Tris·HCl buffer pH 7.5. The samples were transferred to the MaxiSorp plate and incubated shaking for one hour at room temperature. The presence of  $\beta$ -lactamase was detected with Nitrocefin as described in section 8.3.4. Control: 100  $\mu$ l 1:2500 HRP conjugated goat anti-rabbit was added to the other well and incubated for one hour shaking at room temperature. The assay was developed as described in section 8.1.4.

# Appendix A

## Additional figures and tables

### A.1 Figures to chapter 3

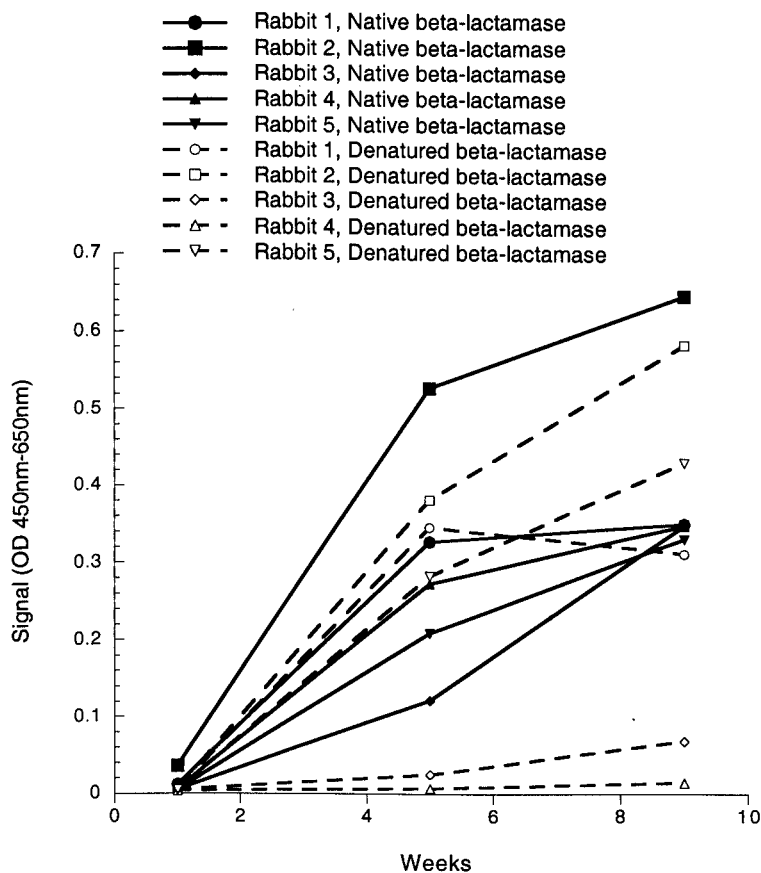


Figure A.1: ELISA results with test-bloods from rabbit 1, 2, 3, 4 and 5. Biotinylated native and denature  $\beta$ -lactamase (308C) was displayed on a streptavidin plate. The  $\beta$ -lactamase was denatured by reducing an internal disulphide bond and carboxymethylate the two cysteines. The sera were diluted 1:25,000.

## **A.2 Figures to chapters 4 and 5**

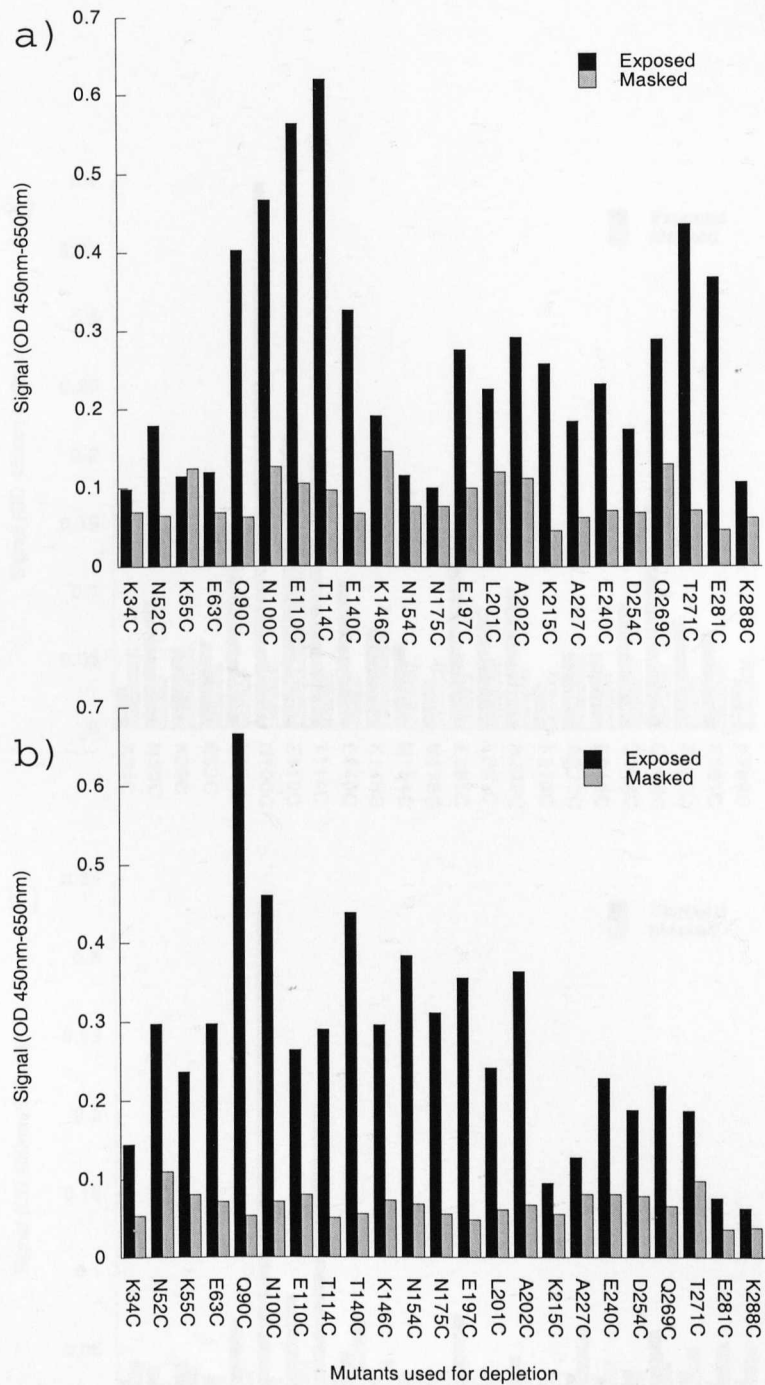
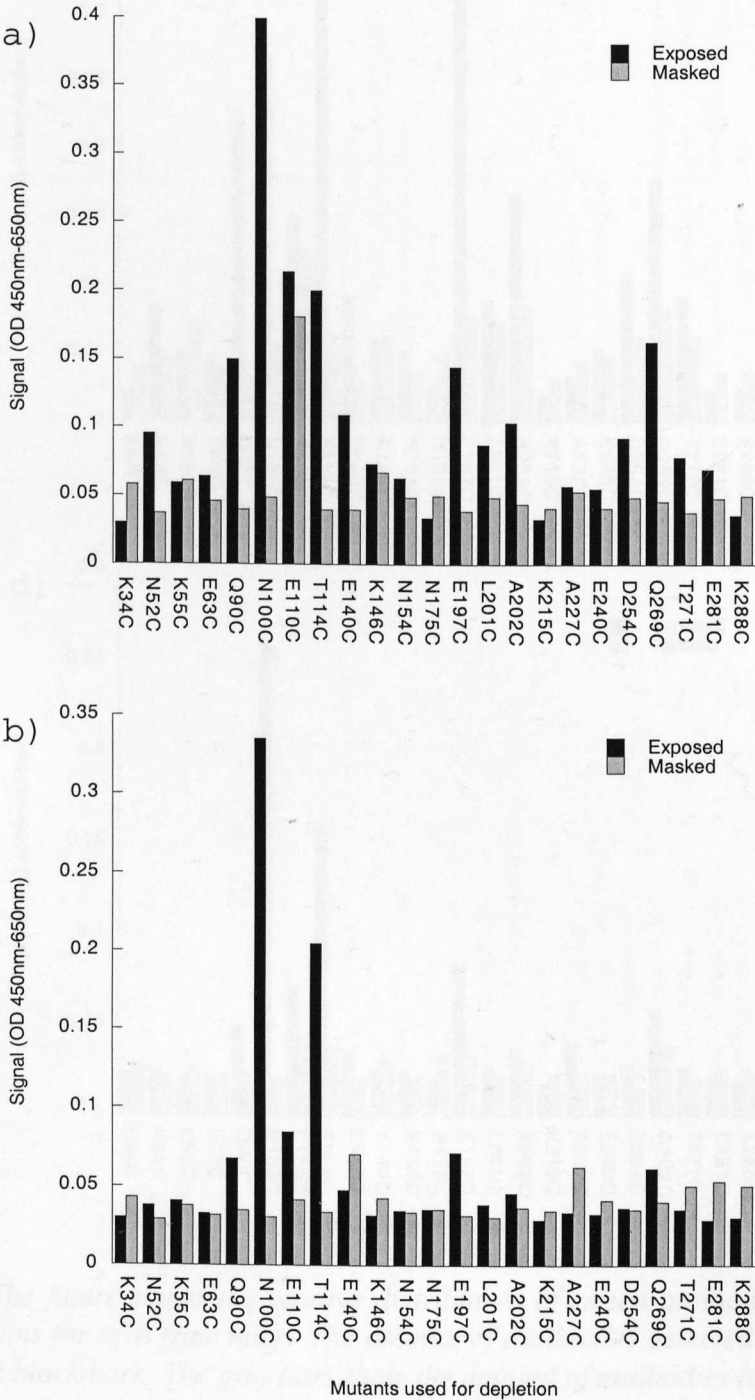


Figure A.2: The figure shows the relative distribution of serum antibodies to different surface locations. The amount of antibodies detected to each region is displayed in black bars. The grey bars show the amount of antibodies to the remaining surface that were not successfully subtracted from the serum. The sera concentrations in the mapping assay were 1:10,000 and 1:50,000 for the rabbits 1 and 2 respectively. The biotinylated antigen captured on streptavidin beads and plates to specifically absorb the serum for antibodies to the unmasked antigen surface. Both rabbits were primarily injected with antigen in FCA followed by antigen in FIA in first and second boost.



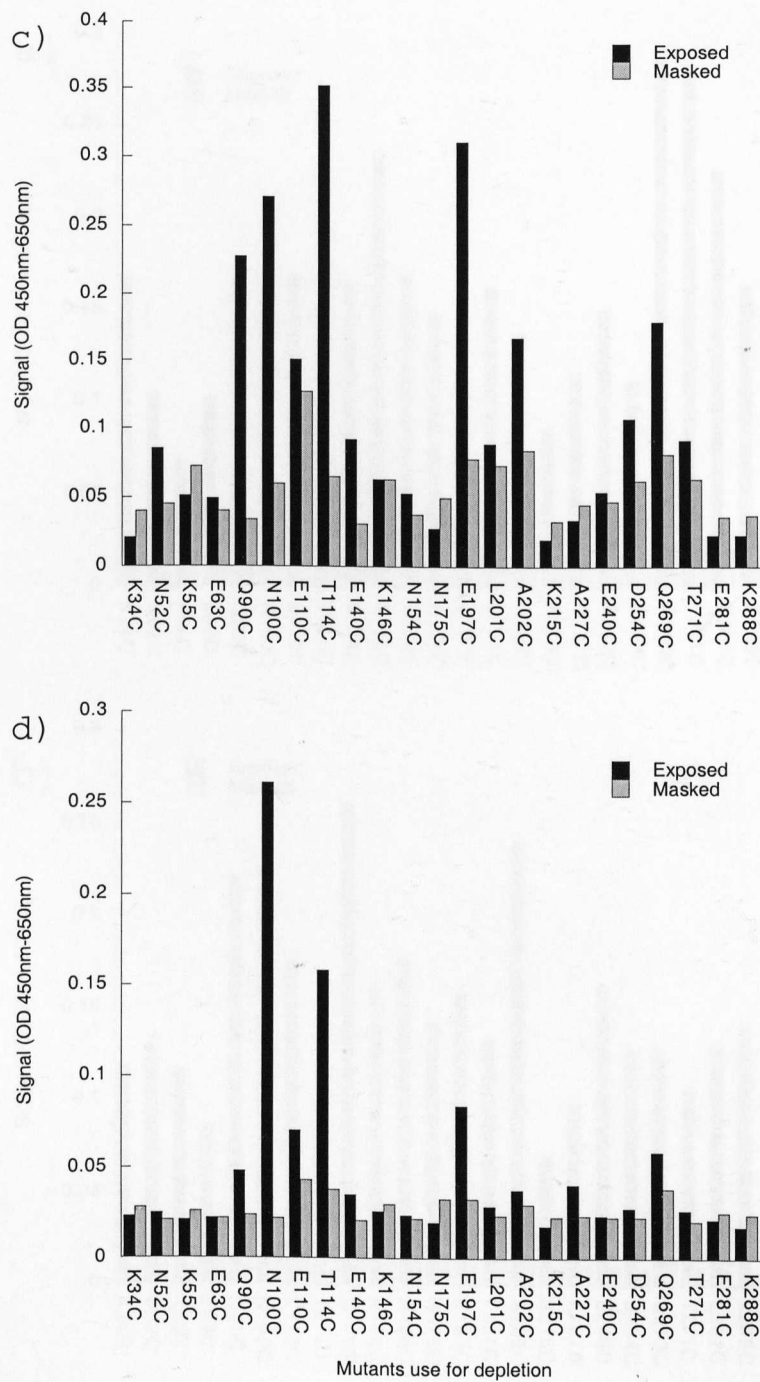


Figure A.3: The figure shows the relative distribution of serum antibodies to different surface locations for sera from mice. The amount of antibodies detected to each region is displayed in black bars. The grey bars show the amount of antibodies to the remaining surface that were not successfully subtracted from the sera. The serum concentration in the mapping assay was 1:5,000. The mice (mouse 1, 2, 3 and four in image (a), (b), (c) and (d) respectively) were primarily injected with antigen in FCA followed by antigen in FIA in first and second boost.



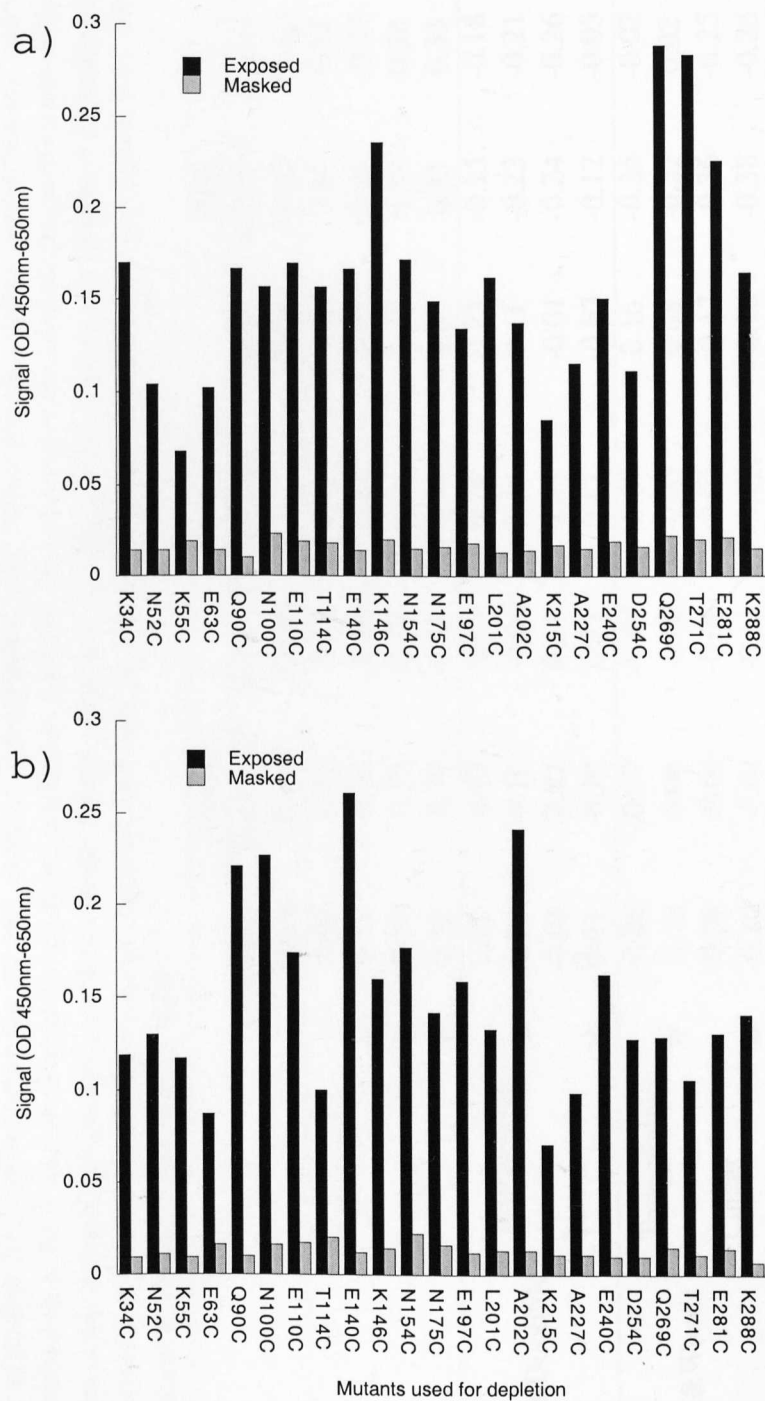


Figure A.4: The figure shows the relative distribution of serum antibodies to different surface locations. The results for rabbit 3 and 4 are shown in image (a) and (b) respectively. The amount of antibodies detected to each region is displayed in black bars. The grey bars show the amount of antibodies to the remaining surface that were not successfully subtracted from the serum. The serum concentration in the mapping assay was 1:30,000. The rabbits were given three injections with antigen in PBS.

Table A.1: Calculated correlation coefficients between relative amount antibodies detected and the surface properties backbone mobility, hydrophilicity and surface accessibility. The data sets are prepared for the 23 cysteine positions. The epitope distributions are based on the data in the figures 3.6, 3.7, 4.1 and 5.1. The B-values of the backbone atoms is used as the backbone mobility. This data set is corrected for contact residues in the crystal structure. The hydrophilicity values are taken from Hopp and Woods [1981] and Kyte and Doolittle [1982]. The surface accessibility is defined as the contact area of a probe of radius 10 Å rolling over the protein surface. Topographic: The values for the surface properties are calculated as average values for surface residues in a 10 Å radius around the cysteine positions. Linear: The values for the surface properties are calculated as average values for seven amino acids around the cysteine positions in the linear sequence. w: The ELISA signals are weighted by using the square values. Beads/Plates: The biotinylated β-lactamase mutants were captured on beads or plates during the depletion step (see figure 3.1). The rabbits were immunised with β-lactamase in Freund's adjuvant or PBS.

		Freund's							PBS			
		Rabbit 1		Rabbit 2*		Rabbit 2		Average	Rabbit 3		Rabbit 4	
		Beads	Beads	Beads	Beads	Plates	Plates		Beads	Beads	Beads	Plates
Backbone mobility	Topographic	0.65	0.55	0.55	0.39	0.39	0.71	0.71	0.16	0.34	0.12	0.33
		w	0.71	0.55	0.39	0.39	0.74	0.74	0.02	0.03	-0.17	0.03
	Linear	0.51	0.53	0.53	0.53	0.71	0.60	0.60	0.06	0.37	0.28	0.28
		w	0.52	0.70	0.71	0.70	0.70	0.70	0.03	0.35	0.33	0.24
Hydrophilicity	Topographic	0.27	0.13	0.16	-0.07	-0.04	0.24	0.24	0.22	-0.15	-0.18	0.06
		w	0.24	0.16	-0.04	-0.04	0.24	0.24	0.11	-0.23	-0.21	-0.07
	Linear	-0.01	0.07	0.07	0.11	0.32	0.03	0.03	-0.01	-0.24	-0.26	-0.16
		w	0.01	0.22	0.32	0.32	0.12	0.12	-0.03	-0.12	-0.05	-0.10
	Topographic	-0.08	0.07	0.06	-0.09	-0.08	-0.02	-0.02	0.16	-0.25	-0.02	-0.05
		w	-0.13	0.06	-0.08	-0.06	-0.05	-0.05	0.08	-0.22	0.02	-0.08
Surface accessibility	Topographic	-0.08	-0.01	-0.01	-0.11	-0.11	-0.06	-0.06	-0.12	-0.36	-0.25	-0.31
		w	-0.19	-0.01	-0.11	-0.11	-0.13	-0.13	-0.16	-0.38	-0.26	-0.35
	Linear	0.36	0.11	0.26	0.05	0.06	0.29	0.29	0.11	-0.18	-0.33	-0.04
		w	0.50	0.11	0.06	0.14	0.46	0.46	-0.02	-0.24	-0.44	-0.17
	Linear	0.19	0.11	0.11	-0.18	-0.18	0.18	0.18	0.05	-0.22	-0.24	-0.11
		w	0.28	-0.15	-0.18	-0.18	0.10	0.10	0.48	-0.18	-0.17	-0.22

Table A.2: Mice 1, 2, 3 and 4. Calculated correlation coefficients between relative amount antibodies detected and the surface properties backbone mobility, hydrophilicity and surface accessibility. The data sets are prepared for the 23 cysteine positions. The epitope distributions are based on the data in figure 4.3. The B-values of the backbone atoms is used as the backbone mobility. This data set is corrected for contact residues in the crystal structure. The hydrophilicity values are taken from Hopp and Woods [1981] and Kyte and Doolittle [1982]. The surface accessibility is defined as the contact area of a probe of radius 10 Å rolling over the protein surface. Topographic: The values for the surface properties are calculated as average values for surface residues in a 10 Å radius around the cysteine positions. Linear: The values for the surface properties are calculated as average values for seven amino acids around the cysteine positions in the linear sequence. w: The ELISA signals are weighted by using the square values. Beads/Plates: The biotinylated β-lactamase mutants were captured on beads or plates during the depletion step (see figure 3.1). The mice were immunised with β-lactamase in Freund's adjuvant.

		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Average
Backbone mobility	Topographic	0.58	0.53	0.72	0.53	0.61
	w	0.49	0.45	0.33	0.48	0.47
	Linear	0.59	0.52	0.72	0.52	0.61
	w	0.45	0.37	0.66	0.35	0.46
Hydrophilicity	Topographic	0.11	0.13	0.08	0.08	0.11
	w	0.12	0.14	0.17	0.09	0.13
	Linear	0.02	0.11	0.02	0.03	0.06
	w	-0.01	0.01	0.05	-0.04	0.00
Surface accessibility	Topographic	-0.21	-0.23	-0.13	-0.27	-0.23
	w	-0.20	-0.22	-0.16	-0.26	-0.23
	Linear	-0.25	-0.25	-0.25	-0.25	-0.25
	w	-0.26	-0.26	-0.26	-0.26	-0.26
Surface accessibility	Topographic	0.49	0.54	0.54	0.53	0.55
	w	0.29	0.44	0.57	0.39	0.45
	Linear	0.36	0.39	0.43	0.36	0.40
	w	-0.04	-0.08	-0.07	-0.07	-0.07

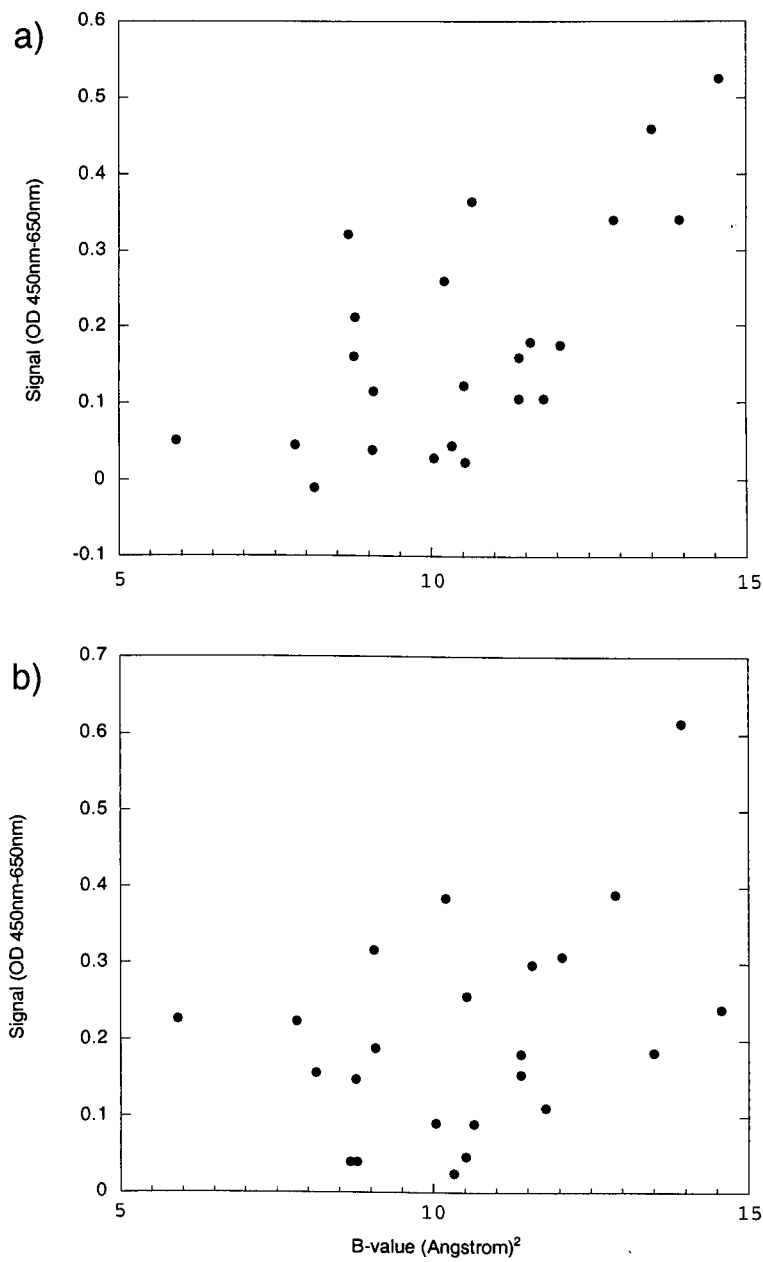


Figure A.5: Rabbit 1 (image a) and rabbit 2 (image b). Scatter plot of the ELISA signals (data in figure A.2) and backbone mobility in the same surface regions (CTRs). The backbone mobility is the average B-value of the surface residues in a radius of 10Å from the cysteine positions.

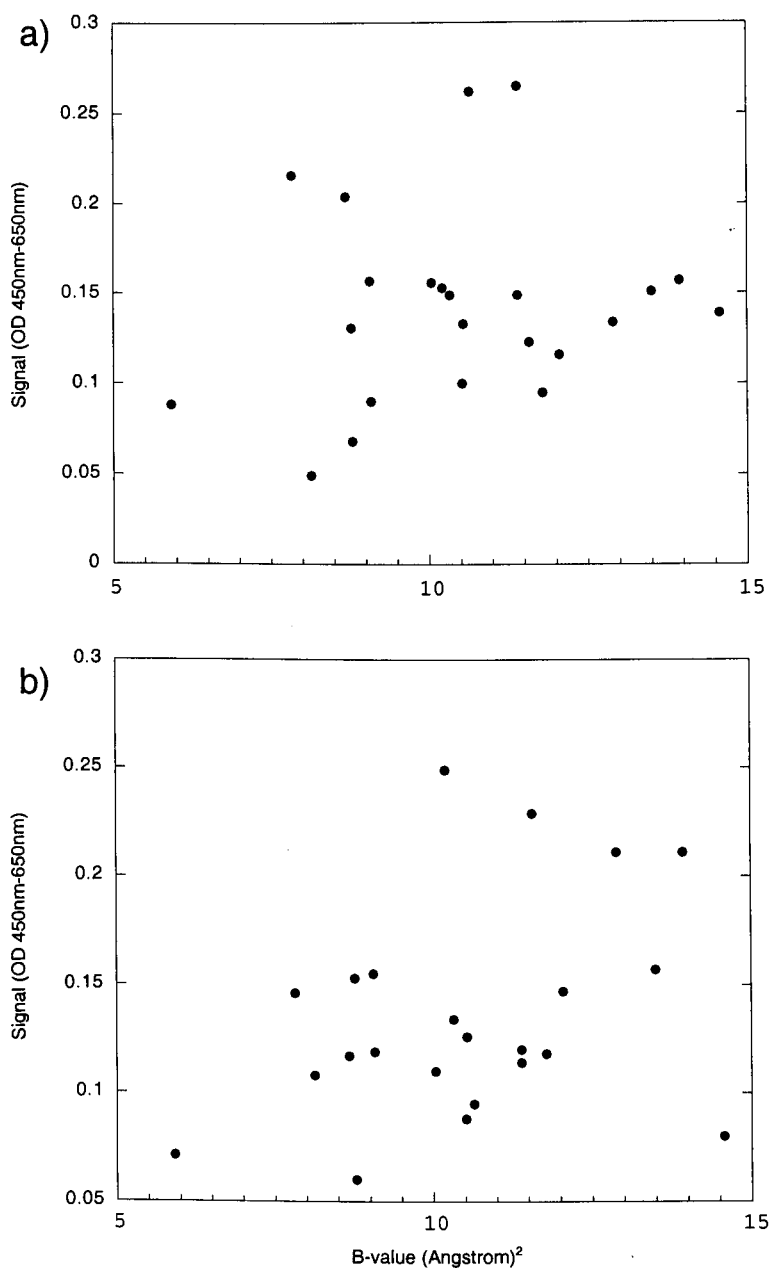


Figure A.6: *Rabbit 3 (image a) and rabbit 4 (image b). Scatter plot of the ELISA signals (data in figure A.4) and backbone mobility in the same surface regions (CTRs). The backbone mobility is the average B value of the surface residues in a radius of 10Å from the cysteine positions.*

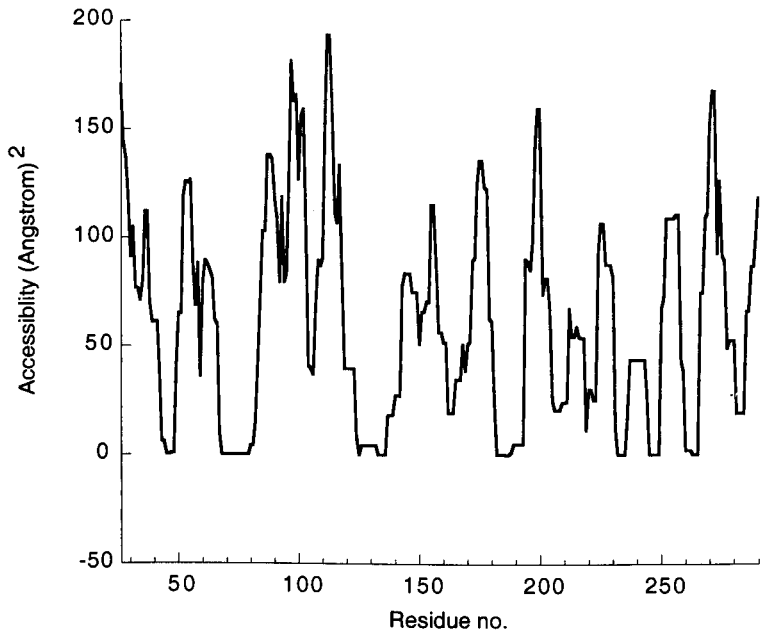


Figure A.7: The accessibility of each residue is set to the contact area of a probe of radius 10Å rolling over the protein surface. The values are average over a window of seven residues along the protein sequence.

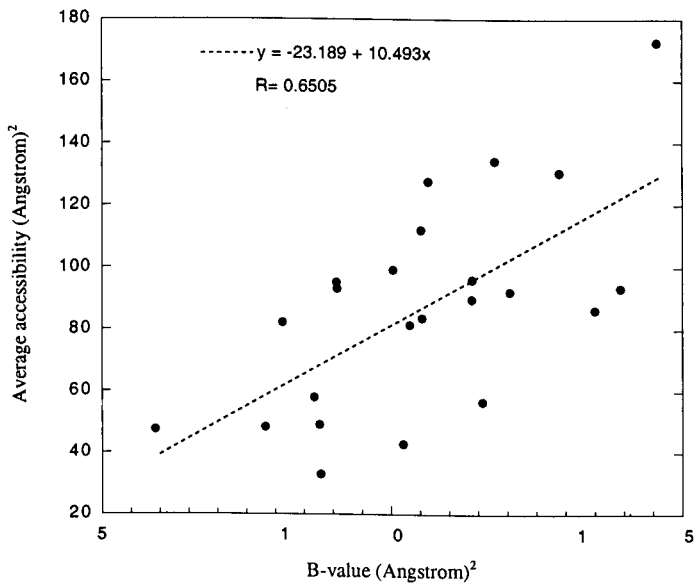


Figure A.8: Scatter plot of backbone mobility and accessibility calculated for each of the CTRs used for epitope mapping. The backbone mobility and accessibility are the average values for residues in a radius of 10 Å around each masking (cysteine) position. The backbone mobility is the B-value of the backbone atoms. The accessibility values are the contact areas of a probe of radius 10 Å rolling over the protein surface. The correlation coefficient is 0.65. The fitted line is given by the equation  $f = 10.49x - 23.2$  with an  $R$  value of 0.65.

## **Appendix B**

### **DNA vector construct**

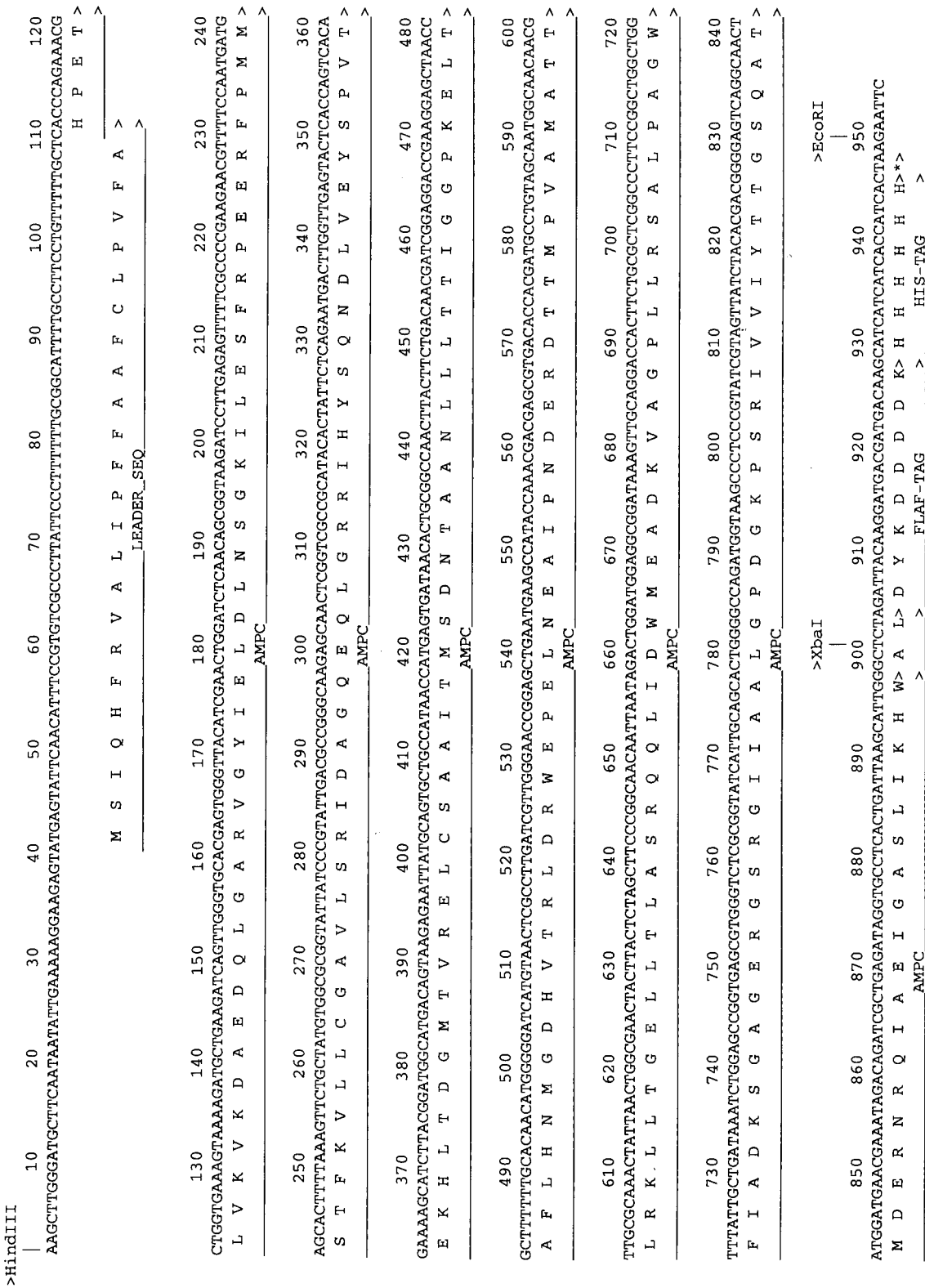


Figure B.1: The figure shows the sequence inserted between the restriction sites HindIII and EcoRI in a modified pUC19 vector. The gene encoding  $\beta$ -lactamase is followed by a FLAG tag, His (6) tag and an ochre stop codon. The sequence includes the ribosomal binding site and leader sequence from pBR322. The pUC19 vector was modified by replacing the ampicillin resistance gene with a gene encoding chloramphenicol resistance.



## **Appendix C**

### **Hydrophilicity models**

Table C.1: *The table shows the hydrophilicity values given each amino acid in the two models Hopp&Woods [Hopp and Woods, 1981] and Kyte&Doolittle [Kyte and Doolittle, 1982].*

Amino acid	Hoop & Woods	Kyte & Doolittle
Alanine	-0.5	-1.8
Phenylalanine	-2.5	-2.8
Lysine	3.0	3.9
Proline	0.0	1.6
Threonine	-0.4	0.7
Cysteine	-1.0	-2.5
Glycine	0.0	0.4
Leucine	-1.8	-3.8
Glutamine	0.2	3.5
Valine	-1.5	-4.2
Aspartate	3.0	3.5
Histidine	-0.5	3.2
Methionine	-1.3	-1.9
Arginine	3.0	4.5
Tryptophan	-3.4	0.9
Glutamate	3.0	3.5
Isoleucine	-1.8	-4.5
Asparagine	0.2	3.5
Serine	0.3	0.8
Tyrosine	-2.3	1.3

# Appendix D

## List of chemicals and materials

Anti-mouse	M-4280	Sigma
Anti-mouse-HRP	A-2554	Sigma
Anti-rabbit	R-2004	Sigma
Anti-rabbit-HRP	A-6154	Sigma
Bayol F	14500.01	Serva
ABI PRISM BigDye terminator mix	4303150	PE Biosystems
Bis-Tris protein gels	NP0302	Invitrogen
Biotin-HPDP	21341 ZZ	Pierce
Biotin-Maleimide	B-1267	Sigma
BSA	A-7906	Sigma
Cyanogen bromide	16774	Fluka
Cytochrome c	C-2506	Sigma
Freund's complete adjuvant	263810	Difco Laboratories
Freund's incomplete adjuvant	263910	Difco Laboratories
ECL	RPN 2106	Amersham Biosciences
Glass Syringe Luer Lock 2ml	SZR285001D	Fisher Scientific
Homogeniser probe S8N-5G	361-118	IKA
HSA	A-3782	Sigma
Iodoacetamide	I-6125	Sigma
Iodoacetic acid	I-2512	Sigma
LDS sample buffer	NP0007	Invitrogen

Lys-C	1047 825	Boehringer
Lysozyme	L-6876	Sigma
Mark12 marker	LC5677	Invitrogen
M2 antibody	F-3165	Sigma
Maxisorp ELISA plates	1989685	Roche
Modified trypsin, sequence grade	1418025	Roche
Nickel-NTA beads	30210	Qiagen
Nitrocefin	484400	Calbiochem
Nitrocellulose membranes	LC2000	Invitrogen
Nunc immunoplates Maxisorp	442404 A	Life Technologies
PD-10 desalting columns	17-0851-01	Amersham Biosciences
PEG 3,000-3,700	P-2906	Sigma
Rapid-endotest	91-103	BioWhittaker
Sanitary adapter 3 way	008212586	VWR International
SeaBlue marker	LC5626	Invitrogen
Streptavel High Bind 96	1989685	Roche
Streptavidin	S-4762	Sigma
Streptavidin beads magnetic	112.06	Dynal
TMB	T-2885	Sigma
Trypsin	T-8642	Sigma
Trypsin, sequencegrade	1418025	Roche
Tween 20	17767-B	NBS Biologicals
X-Omat Film Kodak	306722410	VWR International

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